

EVALUATION OF A TRANSGENIC ZEBRAFISH MODEL FOR ASSESSING
ARSENIC TOXICITY

A Thesis Submitted to the College of Graduate Studies and Research in Partial
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By
Heather Marie Salisbury

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Chair of the Toxicology Group
Toxicology Centre
University of Saskatchewan
Saskatoon, Saskatchewan
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Abstract

The objective of this thesis was to evaluate *hsp70* expression as an indicator of arsenic exposure in zebrafish larvae and to assess the accuracy of the *hsp70*-eGFP reporter gene construct as a reliable indicator of endogenous *hsp70* expression. The relative toxicity of arsenite and arsenate was also examined and gross developmental effects were recorded following an acute 96 hour range finding exposure. Gross effects observed included edema, trunk abnormalities, immobility, and mortality for both arsenite and arsenate, with arsenite more toxic than arsenate. The median lethal concentrations (LC₅₀) for arsenite and arsenate were calculated from the data obtained in the 96 hour exposure. They were determined to be 771.98 μ M and 1347 μ M respectively. The effective concentrations (EC₅₀) were determined to be 570 μ M for arsenite and 1172 μ M for arsenate. Results from the 96 hour exposures were also used to determine concentrations used in subsequent exposures. Induction of *hsp70* was examined in wild-type larvae following a three hour exposure to arsenic and subsequent in situ hybridization. It was determined that both trivalent and pentavalent arsenic induced expression in the olfactory rosette, gills and skin, GIT, liver, and pericardial muscle. Expression was found to be dose-dependent and tissue-specific for both. Induction of *hsp70* was evident in the skin, liver, and gastrointestinal tract of zebrafish larvae exposed to 700 μ M arsenite and in the skin, gills, liver, pericardial muscle, and gastrointestinal tract in those exposed to 1000 μ M or 2000 μ M arsenite. Exposure to 1500 μ M arsenate resulted in expression in skin, liver, and gastrointestinal tract, while induction was observed in skin, gills, liver, pericardial muscle and gastrointestinal tract of larvae exposed to 2500 μ M or 7500 μ M arsenate. Overall

expression patterns of *hsp70*-eGFP in transgenic zebrafish larvae exposed to arsenic were found to closely mimic that of endogenous *hsp70* expression patterns in wild-type larvae suggesting that it is an accurate indicator of endogenous *hsp70* expression.

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Dedication

This is dedicated to the memory of Rena Fraser: my mom, my friend, my champion. Always remembered; always loved.

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List of Abbreviations

Ab.....	antibody
AhR.....	aryl hydrocarbon receptor
AhRE.....	aryl hydrocarbon response elements
Al.....	aluminum
As.....	arsenic
AsIII.....	arsenite (trivalent arsenic)
AsV.....	arsenate (pentavalent arsenic)
BCF.....	bioconcentration factor
BCIP.....	5-bromo-4-chloro-3-indolyl-phosphate
BSA.....	bovine serum albumin
Cd.....	cadmium
Cu.....	copper
CHSE.....	chinook salmon embryo
DIG.....	digoxigenin
DMA.....	dimethylarsonous acid
EC ₅₀	median effective concentration
EDTA.....	ethylenediamine tetra-acetic acid
eGFP.....	enhanced green fluorescent protein
Eh.....	redox potential
EtOH.....	ethanol
Fe.....	iron
GFP.....	green fluorescent protein
GIT.....	gastrointestinal tract
Hg.....	mercury
hpf.....	hours post fertilization
hr.....	hour
Hsc.....	heat shock cognate protein
HSE.....	heat shock element
HSF.....	heat shock transcription factor

<i>hsp</i>	heat shock gene or mRNA
Hsp.....	heat shock protein
<i>hsp70</i> -eGFP.....	<i>hsp70</i> promoter fused to enhanced green fluorescent protein reporter gene
LC ₅₀	median lethal concentration
MMaIII.....	monomethylarsonous acid
MMaV.....	monomethylarsonic acid
NaCl.....	sodium chloride
NaOH.....	sodium hydroxide
NBT.....	4-nitro blue tetrazolium chloride
Ni.....	nickel
Pb.....	Lead
PBS.....	phosphate buffered saline
PBST.....	phosphate buffered saline with Tween-20
PFA.....	paraformaldehyde
ppb.....	parts per billion
ppm.....	parts per million
R ²	coefficient of determination
RTH.....	rainbow trout hepatoma
SDS.....	sodium dodecyl sulfate
SEM.....	standard error of the mean
-SH.....	sulfhydryl group
TCDD.....	2,3,7,8-tetrachlorodibenzo-p-dioxin
U.....	uranium
Ud.....	undetected

1.0 General Introduction

The ever-expanding number of chemicals being released into the environment has necessitated the development of rapid and sensitive assays that can provide some understanding of the potential impact of these substances on organisms. Traditional toxicology assays have relied on endpoints such as mortality, reproduction and growth but these methods can be time consuming and expensive, and offer little or no insight into the toxic mechanisms of the compounds. While these measurements need to be included to understand the total impact of a substance on an organism, a need for rapid and economical methods that offer some understanding of the effects of a substance has prompted more research into molecular or subcellular indicators of toxicity. Biological responses are expressed at the subcellular level initially; therefore, changes at this level may be a more sensitive indicator of toxicity than endpoints such as necrosis or mortality. The quest for a more sensitive assay has led to a collaboration of various scientific disciplines such as ecology, biochemistry and physiology in the development and evaluation of various biomarkers to detect and assess environmental stressors.

Heat shock proteins (Hsps) are one of a number of biomarkers currently being developed and evaluated (de Pomerai, 1996; Bierkens, 2000; Carnevali and Maradonna, 2003). Many aspects of Hsps, such as their ubiquity and high level of conservation, make them attractive as indicators of toxicity. Stress proteins have been identified as being one of the principal defense mechanisms that are activated by the presence of denatured proteins in the cell. Since stress proteins are part of the cell's protective

mechanism, the accumulation of Hsps should be closely linked with the organism's physiological state, providing insight into the effects of toxicants at the cellular level and their ultimate effects on the whole organism. Also, exposure to a wide variety of environmental stressors such as elevated temperatures (Lele *et al.*, 1997), various metals and metalloids including arsenic (Kothary *et al.*, 1982; Lang *et al.*, 2000), and various pesticides (Dyer *et al.*, 1993) can induce cells to increase production of heat shock proteins. This suggests that the heat shock response may be a useful endpoint for detecting diverse environmental stressors, and may be an indicator of further downstream effects. For example, *hsp70* induction in the olfactory epithelium has been demonstrated following waterborne exposure of zebrafish (*Danio rerio*) to toxicants such as cadmium (Cd) (Blechinger *et al.*, 2002a,c). Also, copper (Cu) is known to induce *hsp70* (Boone and Vijayan, 2002; Airaksinen *et al.*, 2003), and exposure to Cd or copper has been shown to result in damage to the olfactory epithelium and impaired olfactory function in fish (Hansen *et al.*, 1999; Scott *et al.*, 2003). Fish detect alarm substance by olfaction and, in response, exhibit predator avoidance behaviors. Waterborne exposure to Cd has recently been shown to result in accumulation of Cd in olfactory neurons and elimination of these normal antipredator behaviors, however, dietary exposure resulted in no change (Scott *et al.*, 2003). This demonstrates a possible link between *hsp70* induction in the olfactory rosette and subsequent behavioral changes that could have an impact on population interactions and species survival.

Arsenic (As) is a widespread environmental contaminant that is found naturally in the earth's crust and as a result of anthropogenic activity. It has increasingly become

a problem as contaminated ground water is being tapped for drinking water in many areas of the world to accommodate the growing population. Relatively small amounts of As pose a variety of health risks for humans as well as for many aquatic organisms. In humans, exposure to As in drinking water has been associated with dermatological effects, various cancers, cardiovascular and neurological effects, and diabetes (Abernathy *et al.*, 1999). In aquatic environments, effects such as reduction in algal biomass, decreases in Daphnid survival and reproduction, and cancer in fish are all possible results of As exposure (Tisler *et al.*, 2002; Eisler, 2004). Exposure to arsenicals *in vitro* and *in vivo* in different model systems has been shown to induce a number of stress proteins including Hsp70 (Del Razo *et al.*, 2001). Therefore, the stress response would appear to be a reliable biomarker of As exposure and effect and the expression level of the protein might indicate the level of exposure.

Standard procedures for predicting the toxicity of agents have, in the past, involved exposing organisms to a maximum tolerated dose and observing for toxic effects. However, relatively recent developments are changing the way some toxicological evaluations can be performed. For example, much research has led to the establishment of transgenic organisms with heterologous reporter genes under the control of stress-inducible hsp-gene promoters. The generation of a transgenic line of zebrafish carrying an inducible *hsp70* promoter fused to an enhanced green fluorescent protein (eGFP) reporter gene was recently accomplished by Halloran *et al.* (2000). The generation of transgenic organisms has made it possible to identify individual cells and tissues affected by a substance in early life stage, intact, living organisms.

Early life stages of fish are generally believed to be the most sensitive to the effects of As, Cd, and a variety of other toxicants (McKim *et al.*, 1975; Strmac and Braunbeck, 1999; Blechinger *et al.*, 2002a,b), and zebrafish offer an excellent vertebrate model to study developmental toxicity. Features of zebrafish embryogenesis have been extensively studied (Kimmel, 1989; Kimmel *et al.*, 1995) and there has been considerable advancement in the understanding of the genetic basis for their development (Driever *et al.*, 1996; Haffter *et al.*, 1996; Thisse and Zon, 2002)). These, and numerous other benefits (see section 1.5), make the zebrafish the vertebrate model of choice in many early life stage studies.

An *in vivo* system that uses the activation of *hsp70* as a measure of toxicity in early life stages of transgenic zebrafish carrying an *hsp70*-enhanced green fluorescent protein reporter gene (*hsp70*-eGFP) was recently established in our lab and was shown to be a reliable indicator of Cd toxicity (Blechinger *et al.*, 2002a,c). Utilizing this *in vivo* system to examine the developmental effects of As exposure allows for toxicant-affected cell identification in a well defined, whole, living organism.

1.1 Hypothesis

The major hypothesis of this study is that *hsp70* expression is a reliable indicator of exposure to arsenic, and that it is induced in a tissue-specific and dose-dependent manner in zebrafish larvae. It is further hypothesized that induction of the reporter gene *hsp70*-eGFP closely mimics endogenous *hsp70* induction in response to As exposure, and that monitoring *hsp70*-eGFP induction should therefore prove to be a rapid and accurate *in vivo* assay method.

1.2 Heat Shock Proteins

All organisms, from bacteria to humans, respond to heat and other physical or chemical stressors by the increased synthesis of an evolutionary conserved family of proteins called heat shock proteins (Hsps). These Hsps were first discovered in 1962 when researchers in Ritossa's lab observed puffs on *Drosophila* salivary gland polytene chromosomes in response to heating (Ritossa, 1962). These were areas of active transcription of genes encoding what were consequently named heat shock proteins (Hsps). Heat shock proteins have since been found to be ubiquitous to all organisms, and amino acid sequences are highly conserved among any one family of Hsps (reviewed in Iwama *et al.*, 1998).

Heat shock proteins are a large family of molecular chaperones that are part of the cellular defense system. Relative molecular mass has been used to classify these proteins into families that are represented in the various compartments of the cell including the cytoplasm, nucleus, mitochondria, chloroplasts, and the endoplasmic reticulum. Recognized families of Hsps include Hsp100, Hsp90, Hsp70, Hsp60, Hsp47, and low molecular weight Hsps that range from 16 to 30kDa (Morimoto *et al.*, 1994). Some Hsps are constitutively expressed in unstressed cells and others, such as Hsp70, are believed to be strictly stress inducible. Constitutive Hsps, known as heat shock cognates (Hscs), act as molecular chaperones to aid in protein folding and assembly, to maintain proteins in unfolded configurations for translocation across membranes, and to prevent inappropriate interactions with other proteins under normal growth conditions (Ellis and Hemmingsen, 1989). These molecular chaperones are not part of the final protein structure but function by recognizing and then repeatedly binding and releasing

the unfolded or malfolded proteins in an ATP-dependent process, assisting protein folding into functional conformations (Ryan and Hightower, 1999). Additional Hsp chaperoning functions are required following exposure of cells to stressors that cause protein denaturation and aggregation. Inducible Hsps are expressed in response to this requirement for additional chaperones in the cell.

Many aspects of Hsps make them attractive as biomarkers of toxicity. Members from at least four of the Hsp families are inducible, but to different degrees and often in different cellular compartments and tissues (Ryan and Hightower, 1999). It is generally believed that Hsp induction is greatest in tissues that are most susceptible to damage caused by a particular stressor but the true potential of the various Hsp families as biomarkers is still unclear and much research is needed to establish their validity. However, the Hsp70 isoform has proven to be the one induced by the widest variety of stressors in the greatest number of species (reviewed by Iwama *et al.*, 1998) and therefore shows the most promise as a broad biomarker of exposure and effect. Since the synthesis of Hsp70 is a broad response induced by a variety of stressors, the accumulation of Hsp70 also has the potential to provide information on the overall impact of multiple stressors on the physiological state of the organism. In the past few decades, an extensive literature base on the fish heat shock response has become available as a result of numerous studies and reviews on the subject.

The cloning and characterization of several of the zebrafish heat shock genes including *hsp47* (Lele and Krone, 1997), *hsp90* (Krone and Sass 1994; Sass *et al.*, 1996) and an inducible *hsp70* gene (Lele *et al.*, 1997) was achieved previously in this lab. Expression during normal development and following environmental stress was

examined for each of these (reviewed by Krone *et al.*, 1997; Krone *et al.*, 2003). One of the *hsp70* genes isolated by Lele *et al.* (1997), the *hsp70-4* gene, was shown by Northern blot analysis to have no expression at control temperatures but was strongly heat-inducible. Lele *et al.* (1997) also found only slight increases in *hsp70* mRNA levels following exposure to ethanol as compared to heat-shock induction, demonstrating that there may be some degree of stressor-specificity.

1.3 Heat Shock Protein 70

1.3.1 General Heat Shock Protein 70

The Hsp70 family is the most extensively studied group of Hsps and they show much promise as biomarkers of not only exposure but also of effect. They are highly conserved across both the plant and animal kingdoms, and are well characterized as molecular chaperones that facilitate protein folding and translocation. At least seven different *hsp70* genes have been identified in mammals, and although expression of the constitutively expressed Hsps is up-regulated in response to stress, only the *hsp70.1* and *hsp70.3* genes are considered highly stress-inducible. In all animals, Hsc70 exhibits a variety of chaperoning duties including folding of nascent proteins and aiding in protein translocation across cellular membranes. The main role of Hsp70, however, is to assist in the refolding of damaged and aggregated proteins following exposure to heat, toxicants, or other types of cellular shock.

Mitochondrial Hsp70 (Grp75) is constitutively expressed in the mitochondrial matrix and assists protein transport across the mitochondrial membranes. This, like Hsp60, may be useful as an organelle-specific biomarker for agents that target mitochondrial proteins (Ryan and Hightower, 1999). The 78 kDa glucose regulated

protein (BiP) is also a member of the Hsp70 family and may be useful as a biomarker of effect for agents that target proteins in the endoplasmic reticulum (ER) and interfere with the folding or processing. Located in the lumen of rough ER, Grp78 facilitates the import of polypeptides and assists in protein folding (Bole *et al.*, 1986; Munro and Pelham, 1986; Kozutsumi *et al.*, 1988). While it is not always induced in response to heat shock, it is strongly induced by glucose deprivation (Shiu *et al.*, 1977), calcium ionophores (Li *et al.*, 1993) and other substances that interfere with the folding and processing of proteins in the ER (Ryan and Hightower, 1999).

1.3.2 Fish Heat Shock Protein 70

A number of studies have examined Hsp induction in fish cell lines and primary cell cultures. Heilkkila *et al.* (1982) examined induction of stress proteins in a fish cell line following heat shock and following exposure to metal ions. Working with CHSE (Chinook salmon embryo) cell lines, they demonstrated that some proteins appear to be preferentially synthesized in response to heat shock (65 and 41 kDa proteins) or metal exposure (51, 46, and 28 kDa proteins), but that others such as 68, 70 and 84 kDa proteins are synthesized to similar extents in response to both heat shock and metal ion exposure. In a similar study, Misra *et al.* (1989) found that Hsp70 was increased in RTH (rainbow trout hepatoma) and CHSE lines following heat shock and exposure to heavy metals. Although cell line studies offer advantages such as simple maintenance and uniformity, caution must be used in extrapolating the results from these experiments to whole fish since these undifferentiated and cancerous lines may not accurately represent the metabolic state of the cells *in vivo*.

In contrast to cell lines, primary fish cell cultures maintain their differentiated characteristics and have been used to further examine heat shock protein expression in fish. An increase in Hsp70 following heat shock and exposure to heavy metals has been demonstrated in a variety of primary cell cultures including desert topminnow (*Poeciliopsis lucida*) hepatocytes (White *et al.*, 1994; Norris *et al.*, 1995), and renal proximal tubule cell cultures of winter flounder (*Pseudopleuronectes americanus*) (Brown *et al.*, 1992). Ryan and Hightower (1994) used polyacrylamide gel electrophoresis to detect changes in cellular stress protein levels in desert topminnow hepatoma-derived cells and winter flounder kidney cell cultures. Following exposure to Cu and Cd, the level of Hsp70 was found to increase with increasing concentrations of stressor in both cell cultures. Renfro *et al.* (1993) also reported induction of *hsp70* in a fish renal tissue preparation following zinc exposure or a mild heat shock. However, although fish cell cultures are more complex than cell lines, they may still be too simple to readily extrapolate results to whole animals.

More recently, whole animal studies have investigated the expression of heat shock proteins in fish. Supporting the findings of cell line and primary cell culture studies, increases in Hsp70 have been demonstrated in a variety of tissues in fish following exposure to elevated temperatures and environmental toxicants (Williams *et al.*, 1996; Janz *et al.*, 1997; Hassanein *et al.*, 1999; Blechinger *et al.*, 2002a,c). Heat shock protein 70 has also been examined in whole, early-life stage zebrafish under control and heat shock conditions as well as following toxicant exposure (Lele *et al.*, 1997; Krone *et al.*, 1997; Blechinger *et al.*, 2002a,b,c). Under control conditions, *hsp70* expression was detected in only the developing lens of the zebrafish larvae. An

increase in expression of *hsp70* throughout the embryo/larvae was detected following exposure to elevated temperature, while exposure to toxicants such as ethanol (EtOH), As, and Cd resulted in increased expression of *hsp70* only in specific cells and tissues. An upregulation of *hsp70* in the olfactory epithelium of zebrafish larvae following exposure to waterborne Cd was demonstrated in a study by Blechinger *et al.* (2002c), and a subsequent study by Scott *et al.* (2003) showed that antipredator behaviours mediated through olfaction were eliminated in trout following exposure to waterborne Cd. This suggests a possible link between *hsp70* induction and longer effects at the physiological and behavioural levels.

In summary, the Hsp70 family members are considered excellent candidates for biomarkers of exposure and effect because they are the best understood and characterized, are highly conserved in both form and function, and their expression is closely tied to proteotoxicity. Also, various studies have shown Hsp70 to be highly inducible by a large variety of stressors in a number of different organisms (reviewed by Iwama *et al.*, 1998). Fish Hsp70 has now been studied in a number of cell lines, primary cell cultures, and in whole fish, resulting in an abundance of information and resources.

1.4 Heat Shock Response: Regulation and Link to Cell Stress

The heat shock response, characterized by increased expression of Hsps, is a result of exposure of cells and tissues to conditions that cause proteotoxicity. This response is a protective and defensive mechanism that enables cells to tolerate changes in the environment. Enhanced heat shock gene expression in response to various stressors is regulated largely at the level of transcription by a multi-gene family of heat

shock transcription factors (HSF). At least four HSF's (HSF 1-4) with distinct roles are present in vertebrate cells (Schuetz *et al.*, 1991; Nakai and Morimoto, 1993; Nakai *et al.*, 1997). Heat shock factor 1 is the functional vertebrate homologue of the HSF found in yeast and in the fly, and is activated by classical stressors such as heat and metal toxicity (Baler *et al.*, 1993). Heat shock factor 1 is present in unstressed cells in the inactive monomeric form and its activity is regulated at the post-translational level through oligomerization, nuclear translocation and hyperphosphorylation (Baler *et al.*, 1993; Morimoto *et al.*, 1994). The model of Hsp70 induction by HSF1 (reviewed in Morimoto *et al.*, 1994) states that the monomeric HSF1 is bound to Hsc70 in unstressed cells. The Hsc70 is in equilibrium between this complex and free Hsc70 that is available to carry out regular chaperone functions such as aiding in the refolding of denatured proteins and assisting in the proper folding of nascent peptides. The HSF-Hsc complex dissociates in response to proteotoxic stress as the free Hsc70 is depleted by the increased concentration of denatured proteins. The free HSF1 monomers associate to form trimers that bind to heat shock elements (HSE) in the promoter region of the *hsp70* genes. Phosphorylation of the HSF1 trimer bound to the HSE results in activation of *hsp70* transcription, and translation of the mRNA results in Hsp70 proteins that aid in protein chaperoning. When there is an excess of chaperones available the Hsc70's are again available to bind to HSF monomers causing the trimers to dissociate thereby stopping transcription of Hsp70. Although somewhat simplified, this model suggests a direct link between *hsp70* and stress-induced proteotoxicity. However, the heat shock response is complex and recent data indicate that Hsp90 as well as other Hsps also play a key role in regulation of HSF1 (Ali *et al.*, 1998; Voellmy, 2004).

Induction of *hsp*-gene transcription in response to cellular stress appears to be a universal response. Inducible *hsp* genes in all living cells of eukaryotic organisms contain HSE's in the promoter region consisting of a highly conserved 5 base pair sequence (5'-nGAAn-3') in repeating arrays with each repeat inverted relative to the adjacent repeat (Amin *et al.*, 1988; Xiao and Lis, 1988). Eukaryotes, from yeast to humans, respond to toxic stress with an upregulation of *hsp* transcription and sequence homology of the *hsp70* gene coding and promoter regions has been demonstrated in a variety of diverse organisms such as *Drosophila* (Pelham, 1982), fish (Kothary *et al.*, 1984), chickens (Morimoto *et al.*, 1986), and humans (Wu *et al.*, 1985).

1.5 The Zebrafish as a Model System

The zebrafish is a small, tropical, freshwater teleost originating from India that has been the subject of scientific study by developmental biologists for many years. Embryonic development of zebrafish is remarkably similar to humans, and since it is a vertebrate, the zebrafish is evolutionarily closer to humans than many other embryo model systems such as insects or worms. As well, resources for genetic studies of zebrafish embryonic development are considerable. The genome sequence is now available (http://www.sanger.ac.uk/Projects/D_rerio/), and a wealth of other resources such as The Zebrafish Information Network (ZFIN) and *The Zebrafish Book* (Westerfield, 1995), render the zebrafish an excellent model for research in various areas.

Currently, the zebrafish model is experiencing a notable increase in popularity as a model system for various types of research including toxicological studies. The zebrafish is a relatively simple vertebrate animal that is easy and inexpensive to

maintain. Adults are small (~3 cm in length) and a large number can be kept in a relatively small space. Zebrafish are generally prolific spawners and under ideal conditions more than 200 embryos can be produced per zebrafish female per week. They also have a short generation time and therefore, numerous experiments can be carried out in a short period of time. The embryos develop rapidly, (from egg to well-developed embryo within about 24 hours) outside the mother's body making them easily accessible for observation and exposure to chemicals. As well, embryos from one female will develop synchronously making it easy to collect and expose many embryos/larvae at a particular and well defined developmental stage (Kimmel *et al.*, 1995). To test the effects of chemical exposure, zebrafish embryos/larvae at the same developmental stage can simply be placed in water containing the test compound. Techniques such as whole-embryo histochemistry and whole-embryo *in situ* hybridization (Jowett and Lettice, 1994) can then be performed on the embryos/larvae to assess tissue-specific mRNA and protein expression. The size and transparency of the embryos also allow for observation of most cells, even at advanced stages of development, and the entire developmental process can be easily followed.

1.6 Transgenic Zebrafish as Biosensors

In situ hybridization is a frequently used method for detecting tissue-specific mRNA expression in whole organisms. This method allows accurate detection of gene expression only after the organism has been fixed in paraformaldehyde and subjected to a lengthy procedure encompassing several days and numerous steps. Therefore, not only does *in situ* hybridization require sacrificing the organism but also increases the chance of introduced error and provides little insight into possible effects of a toxicant

in the developing organism subsequent to analysis. Although this method is still widely used, the development of transgenes has resulted in more simple and rapid techniques for detecting gene expression.

Transgenes, consisting of a reporter gene such as *lacZ* fused to a promoter from a gene of interest, allow for easier observation of gene expression *in vivo*. Reporter genes encode easily assayed proteins such as luciferase or GFP and replace coding regions of the gene of interest. Promoter regions are made up of specific DNA sequences located upstream from the point of initial transcription. The basal or core promoter, located within approximately 40 base pairs from the transcription start site, is found in all protein-encoding genes. It contains DNA sequences, such as the TATA box, that act as binding sites for the transcription complex made up of a number of general transcription factors and RNA polymerase II. Binding of the transcription complex to the basal promoter initiates low levels of transcription. Further upstream promoter sequences bind specialized transcription factors and play a more significant regulatory role in determining which genes will be expressed in a given cell or tissue type under certain conditions as well as the rate of transcription. Thus, the level of expression of a reporter transgene can be used as an indicator of the level of activity of the promoter to which it is linked.

Many recent studies of gene expression have utilized transgenic cell lines or organisms to observe gene induction following exposure to a variety of toxicants. For example, a number of studies have examined transgenic nematodes (*Caenorhabditis elegans*) with a stress-inducible *lacZ* reporter gene as biosensors of toxic exposure in aquatic environments (Mutwakil *et al.*, 1997; Guven *et al.*, 1994). Mutwakil *et al.*

(1997) determined that transgenic nematodes carrying a *lacZ* reporter gene fused to a stress-inducible *hsp70* promoter, were measurably stressed following exposure to river water polluted with environmentally relevant concentrations of heavy metal. A similar study by Guven *et al.* (1994), also found dose-dependent transgene expression in transgenic nematodes following exposure to heavy metals. A variety of vertebrate models have also been used in transgenic studies. Wirth *et al.* (2002) demonstrated stressor-dependence of *hsp70-1* induction in lungs using a line of transgenic mice carrying a firefly luciferase reporter gene fused to an *hsp70-1* promoter. Also, Mattingly *et al.* (2001) recently utilized zebrafish embryos that had been transiently injected with an AhRE (aryl hydrocarbon response element)-GFP construct and then exposed to TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) to detect AhR (aryl hydrocarbon receptor) function, and to determine whether reporter gene expression accurately predicted sites of TCDD-induced dysmorphogenesis during development. Unfortunately, the transient injection assays used in this and other studies pose a problem for reproducibility. Because the transgene is not passed to offspring (single generation expression), only a portion of the cells will carry or express the reporter-gene construct resulting in a mosaic pattern of expression between different individuals.

Recent advances in transgenic research have resulted in production of stable germline transgenic zebrafish capable of passing the reporter gene to future generations. (Amsterdam *et al.*, 1995; Halloran *et al.*, 2000). For example, the promoter region of the heat-inducible *hsp70-4* gene linked to a green fluorescent protein was used in the establishment of stable germline transgenic zebrafish (Halloran *et al.*, 2000). Green fluorescent protein, originally isolated from the jellyfish *Aequorea Victoria*, is proving

to be a useful reporter for monitoring gene expression *in vivo*. Blechinger *et al.* (2002a,c) successfully utilized this line of transgenic zebrafish carrying the *hsp70*-eGFP construct to demonstrate that this reporter gene is a reliable and dose-dependent indicator of exposure to Cd. This stable line of transgenic zebrafish has been maintained over many generations and offers numerous advantages over models used in the studies mentioned in the previous paragraph. The *hsp70* promoter-driven reporter gene (*hsp70*-eGFP) is advantageous because eGFP can be detected *in vivo* in living embryos/larvae by fluorescence microscopy, eliminating the need to incubate with secondary reagents such as dyes or antibodies. Because the reporter-gene construct has been stably integrated, each cell (somatic or germ cell) has the potential of expressing the gene and it can be passed to future generations. The stable transgenic line also allows for the assessment of effects into maturity following stress-induced gene activation in the embryo or larvae and reduces or eliminates the sacrifice of test animals. Also *hsp70* mRNA has been shown to be highly transient following heat shock (Lele *et al.*, 1997), whereas the eGFP appears to be relatively stable and can still be detected up to ten days following heat shock. This provides an advantage over conventional *hsp70* mRNA detection methods as the time frame for detection of toxic exposure is extended for days even after the stressor has been removed (Blechinger 2002a). This *hsp70*-eGFP reporter gene has proven to be a reliable indicator of toxic exposure to Cd (Blechinger *et al.*, 2002a,c) and appears to be a reliable indicator of AsIII exposure (Blechinger 2002a).

1.7 Introduction to Arsenic

Arsenic is a widespread environmental contaminant coming from both natural sources and human activity such as mining, smelting of non-ferrous metals, burning of fossil fuels, preservation of timber, and past use of As-containing pesticides. Ferguson and Gavis (1972) as well as Cullen and Reimer (1989) have written extensive reviews on As from which the information in this paragraph was obtained. Arsenic is a metalloid and in an aquatic environment can exist in four valency states: -3, 0, +3, and +5. Arsenic cycling in the environment is a complex process, and some arsenite (AsIII) and arsenate (AsV) can interchange oxidation state depending on redox potential (Eh), pH, and biological processes. However, AsIII is generally the prevalent form under reducing conditions and AsV is the stable form in oxygenated environments. Elemental arsenic and arsine (-3) usually exist only in strongly reducing environments. Total As in surface waters usually measures <10 µg As/L, however, levels can range from 250 µg As/L to >5,500 µg As/L in waters impacted by mining (ASTDR, 2000; Eisler, 2004). Organic As species (arsenobetaine) are present in high levels in aquatic organisms as a result of biogenesis of inorganic forms but have no obvious adverse effects in these organisms.

Arsenic is generally well absorbed from the gastrointestinal tract (GIT), skin and gills, and once absorbed is rapidly distributed throughout the body (Sorensen, 1976; Sorensen *et al.*, 1979). Arsenic levels are generally found to be the highest in the liver, kidney, spleen, and gallbladder (Sorensen, 1976; Sorensen *et al.*, 1979; Pedlar and Klaverkamp, 2002), however, intravenous administration of sodium arsenite has also been shown to induce stress proteins in other organs and tissues, such as the heart and

the small intestine, suggesting that these may also be targets of As toxicity (Brown and Rush, 1984). In most species, metabolism of As is characterized by a series of redox reactions and methylations with S-adenosyl methionine (SAM) as the methyl donor and glutathione (GSH) as an essential co-factor (Abernathy et al, 1999). Biotransformation occurs mainly in the liver via the following pathway:



The methylated end products, monomethylarsonic acid (MMAV), monomethylarsonous acid (MMAIII), and dimethylarsonous acid (DMA), are carried to the kidney and are readily excreted in urine. Previously, these methylated metabolites were thought to be less reactive, less acutely toxic, and less cytotoxic (NRC, 1999). However, in a recent study by Petrick *et al.* (2000), it was demonstrated that MMAIII is more toxic than AsIII in Chang human hepatocytes. Using three separate assay methods to test for cell damage, they determined the following order of toxicity: MMAIII > AsIII > AsV > MMAV = DMAV. Subsequently, an *in vivo* mammalian study also demonstrated the higher toxicity of MMAIII in hamsters when administered intraperitoneally (Petrick *et al.*, 2001). These studies challenge the hypothesis that methylation of inorganic As is a detoxification process.

Although the toxic mechanisms of action of As are not fully understood, it is thought that trivalent compounds are the primary toxic forms. A number of sulfhydryl-containing proteins and enzyme systems have been found to be altered by exposure to AsIII, thereby affecting energy production pathways. It has an affinity for vicinal thiol groups and if it binds to a critical thiol or dithiol, the enzyme may be inhibited. Pyruvate dehydrogenase is one of the many enzymes AsIII inhibits and the acute

toxicity of AsIII may be in part from depletion of carbohydrates from the organism because of the effect on gluconeogenesis (Szinicz and Forth, 1988). Although the pentavalent compounds are phosphate analogues and can compete with phosphate in glycolysis or can uncouple oxidative phosphorylation by substituting for phosphate in the mitochondria (Gresser, 1981), the toxicity of AsV is mainly due to its reduction to AsIII.

The induction of Hsps as a result of exposure to As has been demonstrated in a number of studies. However, many of these studies have relied on cell cultures or tissue extracts and few have focused on tissue specificity in whole animal models. For example, Tully et al (2000) demonstrated a significant dose-dependent induction of *hsp70* in HepG2 cell lines following exposure to AsV, Cd, and chromium (Cr). Dyer *et al.* (1993), investigated Hsp expression in gill and muscle tissue from sacrificed fathead minnows (*Pimephales promelas*) and found a significant increase of Hsp70, as well as other Hsps, in these tissues following exposure to sodium arsenite. Rossi *et al.* (2002) examined expression of *hsp27*, *hsp60*, *hsc70*, and *hsp70* mRNA and protein in a cultured human urothelial cell line (UROtsa) following exposure to sodium arsenite. It was determined that of the four, only *hsp70* mRNA and protein were consistently increased in response to the exposure, suggesting that *hsp70* would be a good candidate for a biomarker of As exposure. In the present study, the *in vivo* tissue-specific expression of *hsp70* as a result of exposure to AsIII and AsV was investigated in whole, living transgenic zebrafish larvae.

1.8 Objectives

The following research objectives were implemented in order to address the hypotheses as presented in Section 1:

- 1) Comparison of expression patterns of endogenous *hsp70* and reporter *hsp70*-eGFP in zebrafish larvae following acute exposure to AsIII to assess tissue-specificity and dose-dependence and to determine the relationship to classical indicators of exposure such as the EC₅₀ and LC₅₀.
- 2) Comparison of expression patterns of endogenous *hsp70* and reporter *hsp70*-eGFP in zebrafish larvae following acute exposure to AsV to assess tissue-specificity and dose-dependence and to determine the relationship to classical indicators of exposure such as the EC₅₀ and LC₅₀.
- 3) Exposure of transgenic strains of zebrafish to field samples of waters containing mine tailings. As well, exposures with reference waters from the same area and As exposures corresponding to concentrations found in the contaminated water.

2.0 Materials and Methods

2.1 Adult Care and Embryo Collection, Care and Maintenance

The procedure followed for breeding and maintenance of zebrafish in this study has been established and is outlined in *The Zebrafish Book* (Westerfield, 1995). Adult wild-type fish from Speers Seed Store Ltd. in Saskatoon and transgenic zebrafish were maintained in 56.75 litre (15 gallon) or 38.75 litre (10 gallon) glass aquariums (Hagen, Montreal) at 28.5°C on a light cycle of 14 hr light:10 hr dark. The 14:10 photoperiod keeps the adults in a continual spawning stage. Each aquarium contained between 15-30 zebrafish with roughly equal numbers of males and females. The zebrafish adults were fed a mixture of frozen blood worms (San Francisco Bay Brand, CA) and dried flake food (Nutrafin, by Tetra) at least once per day.

Embryos were collected according to standard procedures (Westerfield, 1995). Plastic containers covered with mesh were placed in the bottom of the aquariums the evening before the embryos were collected. Breeding began each morning at the start of the light cycle. Fertilized eggs fell through the mesh into the underlying container and were collected later that morning. Breeding success varied greatly yielding anywhere from 0-1000 eggs but most collections yield between 200-500 eggs per aquarium for wild-type and 0-100 per aquarium for the transgenic zebrafish. Embryos were divided into 25 ml sterile petri dishes with approximately 50 embryos per dish. To prevent the growth of mold or bacteria, the embryos were cleaned with dechlorinated tap water twice per day and waste matter was removed. Embryos were subsequently

maintained at 28.5°C in an incubator on a 14 hr light:10 hr dark photoperiod, inspected frequently under a dissecting microscope, and any dead embryos or unfertilized eggs were removed. Mortality rates during the first 24 hours of development were typically about 10% and fell to near 0% in the following days. Embryos were staged according to a staging guide by Kimmel *et al.* (1995).

Hatching of the embryos generally began on the third day of development and by 72 hours post-fertilization (hpf) nearly all had hatched. To reduce the growth of mold and bacteria, shed chorions were removed with regular water changes. Post-hatch larvae normally begin feeding between the sixth and eighth day (Westerfield, 1995) as their larval yolk sac is nearly depleted by this time; therefore, maintaining larvae beyond this point requires feeding.

2.2 Fixation and Dechoriation

Fixation of larvae was carried out in 1.5ml microfuge tubes containing 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS) with a maximum number of 20 embryos or larvae per tube. These were then incubated overnight at 4°C and several washes of PBST (phosphate buffered saline (PBS) with 0.1% Tween-20 detergent) were performed the next morning. Manual dechoriation, when necessary, was executed under a low power dissecting microscope using fine forceps. Embryos were then used immediately for *in situ* hybridization or were stored in 100% methanol at -20°C for up to one month.

2.3 Mounting and Sectioning

After washing in triple distilled water, larvae to be sectioned were oriented and embedded in 1.5% agarose gel. The gel was cut into blocks around the larvae and then

dehydrated in 100% ethanol overnight. The following morning the blocks were processed for embedding in JB-4 methacrylate (Polysciences Inc., Warrington, PA) according to manufacturer's instructions. The polymerized blocks were sectioned to 5-6 μm thickness using a glass microtome and resulting sections were then mounted on slides and cover slipped with a xylene based mounting media (Cytoseal XYL).

2.4 Photography

Post-hybridized larvae were either used for whole mount microphotography or were first mounted and sectioned (as described in section 2.3). Larvae for whole mount microphotography were first washed in a solution of 0.02 M HCl for two minutes to dissolve any magnesium-phosphate crystals that had precipitated in the hybridization procedure. This was followed by several rinses with triple distilled water and a 10 minute dehydration in absolute ethanol. Ethanol was then pipetted from the tube and any remaining was soaked up with the corner of a Kimwipe. Larvae were then left to air dry for 1 min. followed by immersion in clearing solution containing 2:1 benzyl alcohol: benzyl benzoate for at least ten minutes in a dark place. The clearing solution causes the tissue to become transparent thereby allowing clear viewing under the compound microscope.

A Nikon Coolpix digital camera mounted on a Nikon Eclipse E600 photomicroscope was used to capture images of sections on slides and whole larvae. Images of the transgenic zebrafish were also captured using this equipment and the GFP fluorescence was detected using a Nikon Y-FL Epifluorescence attachment. All images were processed using Adobe Photoshop 4.0.

2.5 Water and Treatment Solutions

The water used for raising the zebrafish and for preparing treatment solutions was carbon filtered water from the Saskatoon municipal water system. Sodium meta-arsenite (NaAsO_2 , CAS# 7784-46-5) with a purity of 98.7% was obtained from J.T. Baker Inc. (Phillipsburg, N.J.). Arsenate (arsenic acid) in the form of sodium salt heptahydrate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$, CAS# 10048-95-0) with a minimum purity of 98%, was obtained from Sigma Chemical Co. (St. Louis, MO). The solutions for treatments were diluted from stock solutions of 100mM arsenite (As III) or arsenate (As V) in triple distilled water. Samples of effluent discharge were obtained from the Giant Gold Mine in the Northwest Territories along with water from Giant Mine Baker Creek outflow.

2.6 Detection of Endogenous Heat Shock Protein 70 Expression Using *in situ* Hybridization

2.6.1 Background

In situ hybridization is a technique used to determine the presence of a particular RNA or DNA sequence in a cell by hybridizing a complimentary probe to that sequence. In this case *hsp70* messenger RNA (mRNA) was detected and visualized with a complementary (antisense) labeled probe and subsequent immunodetection. The procedure involved the preparation of a digoxigenin (DIG)-labeled antisense RNA probe that was then incubated with permeabilized larvae. When the probe enters the cells it hybridizes, or binds, to the *hsp70* mRNA. Incubation with Anti-DIG, an antibody (Ab) specific for the probe's label, produces a hybrid-Ab complex. The antibody is coupled to an enzyme (alkaline phosphatase) that produces a colored

product when reacted with certain reagents. Cells or tissues expressing the gene of interest can then be identified from the color produced as a result of this reaction.

2.6.2 Probe Synthesis

The *hsp70* antisense probe used in this study was synthesized from the *hsp70*-4 PCR-amplified fragment (642 base pairs) initially cloned in our lab by Lele et al (1997). The plasmid containing the fragment was prepared using a standard alkali lysis procedure. A liquid culture of XL-1 Blue *E.coli* containing the plasmid vector pBluescript II in YT broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, tap water) with 100 µg/ml of ampicillin was incubated overnight in a shaking water-bath at 37°C. Cultures were then placed on ice for 10 min. to cool before transferring 1.0 ml aliquots into 1.5 ml microfuge tubes. Cells were pelleted by centrifugation at 12, 000g for 1 min. and remaining media was completely removed. The pellet was then resuspended in 200 µl of Cell Resuspension Solution (50 mM Glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) using a pipette to facilitate resuspension. Following incubation at room temperature for 5 min. freshly made Cell Lysis Solution (0.2 M NaOH, 1% SDS) was added and mixing was achieved by rapid inversions of the tube. Lysed cell suspension was then put on ice for 5 min. after which 200 µl of ice cold Neutralization Solution (1.32 M potassium acetate pH 4.8) was added. Mixing was facilitated by inverting and flicking the tube. Following a 5 min. incubation on ice, the cell suspension was centrifuged for 5-10 min. at 12,000g. Supernatant containing the plasmid was transferred to a new 1.5 ml microfuge tube and cleaned with a phenol:chloroform extraction. To precipitate the DNA, 1 ml of 95% ethanol (EtOH) was added and the suspension was left at room temperature for 10 min. Supernatant

was then centrifuged for 10 min at 12,000g and ethanol was removed by aspiration. Ice cold 70% EtOH was added before vortexing and subsequent centrifugation for 5 min at 12,000g. After removing EtOH by aspiration, 500 µl of ice cold 70% EtOH was again added and process was repeated. The final pellet was allowed to dry for 15 min. after which it was resuspended in 100 µl of triple distilled water and stored at -20°C.

To linearize the plasmid, a *Bss* H II (GibCo Labs) digest was performed on the plasmid according to manufacturer's instructions. Briefly, the *Bss* H II digest yields a 815 bp fragment containing the hsp70-4 template. The purified plasmid was first thawed and 20 µl was transferred to a clean 1.5 ml microfuge tube. To this 3 µl (10% of final volume) of buffer was added, 2 µl of *Bss* H II, and 5 µl of water to give a final volume of 30 µl. After mixing (with a pipette) and a pulse spin, tube was placed in incubator for 2-3 hr. at 50°C. Following incubation, 1 µl of RNase A was added and the tube was left at room temperature. for 20 min. Triple distilled water was then added to bring volume up to 500 µl and a phenol/chloroform extraction was performed. The cut DNA was precipitated with 2X volume of 95% EtOH and tubes were placed in -20°C freezer to precipitate overnight.

In vitro transcription was performed using T7 RNA polymerase (Life Technologies) to synthesize digoxigenin-11-UTP (Boehringer-Mannheim) labeled antisense cRNA probe from the linearized template. First, 5µl 5x T7 RNA polymerase buffer (GibCo), 1 µl RNAsin , 2 µl 10 mM DTT (GibCo), 1 µl T7 RNA polymerase (GibCo), and 2 µl of 10x DIG labeled nucleotide mix (10 mM ATP, CTP, GTP, 6.5 mM UTP, 3.5 mM DIG-UTP (Boehringer-Mannheim) was added to 10 µl of 10 µg/ml DNA template and was mixed by flicking the tube. After incubation for 1hr at 37°C, an

additional 0.5 µl aliquot of T7 RNA polymerase was added and incubation continued for another hour at 37°C. Subsequent to final incubation, RNA was precipitated by adding 2 µl (1/10X volume) of 0.2 M EDTA, 2 µl (1/10X volume) of 4 M LiCl, and 60 µl (21/2X volume) 95% EtOH and incubating in -20°C freezer overnight. The following day tubes were centrifuged for 30 min at 4°C and pellet was washed in ice cold 70% EtOH and then centrifuged for another 15 min. After removing all EtOH, the RNA pellet was air dried at room temperature for 25 min. and then pellet was redissolved in 20 µl of triple distilled water. RNA probe was stored at -80°C.

2.6.3 *In Situ* Hybridization and Immunodetection of Labeled Probe

The *in situ* hybridization protocol was performed according to the procedure described by Jowett (1997) with modifications (Yoav Gothliff, personal communication). All incubations and washes were performed in 1.5ml microfuge tubes using a volume of 1ml of solution and a maximum of 20 larvae per tube. Fixed larvae that had been stored in methanol were first rehydrated in a graded series of 5 min. washes in methanol:PBST (3:1, 1:1, 1:3) followed by washing in PBST. Following rehydration, the larvae were permeabilized by incubation with a solution of proteinase K (Boehringer-Mannheim) in PBST. Developmental age of the larvae, ascertained using the Kimmel staging guide (Kimmel *et al.*, 1995), determined incubation times in Proteinase K solution: 20 min. for post-hatch larvae, 10 min. for embryos 48-72 hours post fertilization (hpf), 5 min. for 24-48hpf, and no incubation for embryos less than 24hpf. Two 5 min. washes in PBST were followed by post-fixing with 4% paraformaldehyde (Sigma) for 30 min. and then two more washes in PBST. Larvae were then washed in hybridization buffer consisting of 50% formamide, 5X SSC, 5mM

EDTA, 0.1% Tween-20 (Sigma), 50µg/ml heparin (Sigma), 100µg/ml tRNA, and triple distilled water to final volume preferred. This was followed by a 4-6 hour incubation in fresh hybridization buffer at 65°C. Fresh hybridization buffer was then preheated to 65°C to which denatured (2-3 min. at 85°C and 2 min. on ice) *hsp70* cRNA was added at a concentration of 1µg probe/ml buffer and larvae were incubated in 500µl of the solution in a 65°C water bath overnight (16-20hrs). Unbound probe was removed in a series of 10 washes: 3 washes for 10 min. in 3:1, 1:1, and 1:3 ratio of Hybridization Solution:2X SSC solution; 1 x 10 min. wash in 100% 2X SSC; 2 x 30 min. washes in 0.2X SSC; 3 x 5 min. washes in 3:1, 1:1, and 1:3 0.2X SSC:PBST; 1 x 5 min. wash in 100% PBST.

In order to decrease non-specific binding of the Anti-DIG antibody, larvae were incubated on a rotary shaker for 3-5 hours at room temperature in a blocking solution made up of 2% fetal calf serum, 2mg/ml BSA (Sigma), and PBST to a final volume of 10ml. To localize the *hsp70* mRNA which was hybridized to the DIG-labeled probe, the blocked larvae were incubated in a 1:5000 dilution of alkaline phosphatase conjugated Anti-DIG Fab fragments (Boehringer-Mannheim), an antibody specific for DIG, in blocking solution for 5 hours on a rotary shaker. Unbound antibody was removed by four washes in PBST at 4°C (three for 15 min. and one overnight), a 15 min. wash in PBST at room temperature, and a 5 min. wash in triple distilled water. Staining took place at room temperature using NBT/BCIP as substrates for the alkaline phosphatase conjugated Ab-hybrid complex. Larvae were first washed twice for 5 min. in pre-staining buffer composed of 100 mM Tris (pH 9.5), 100 mM NaCl (EMD Chemicals), 50 mM MgCl₂ (Sigma), 0.1% Tween-20, and triple distilled water to 10 ml.

Larvae were then incubated in the staining solution (250 µg/ml BCIP, 250 µg/ml NBT (Roche), 1 mM Levamisol (Sigma), pre-staining buffer to 5 ml) on a rotary shaker at room temperature in a dark area. Staining time varied from 20 min. for early embryos up to 5 hr for post-hatch larvae therefore embryos/larvae were examined periodically until a strong colorimetric signal was evident in treatment groups. Any one stage was stained for the same length of time. When a strong signal was evident, larvae were washed in PBST (2 X 5 min.) at room temperature and fixed in 4% paraformaldehyde for 30 min. Post-fixed larvae were again washed in PBST (2 X 5 min.) and were stored in PBS with 0.02% sodium-azide at 4°C if storing for a period of time.

2.7 Heat Shock Protein 70-Enhanced Green Fluorescent Protein Transgenic Zebrafish

2.7.1 Generation of Stable Transgenic Zebrafish Strain

Our lab was involved in the development of a stable line of transgenic zebrafish (Halloran *et al.*, 2000). This line of zebrafish has an *hsp70*-eGFP reporter gene construct which allows observation of *hsp70* expression in the living organism. The reporter gene construct consists of an inducible *hsp70* promoter region containing the regulatory heat shock elements (HSE) needed for transcription to occur, fused to the coding region for the GFP gene isolated from the fluorescent jellyfish *Aequorea victoria*. The stable line of transgenic zebrafish was created by microinjecting linearized *hsp70*-eGFP into fertilized embryos. Following pairwise mating and subsequent screening, transgenic strain 57 was identified (Halloran *et al.*, 2000). This strain appears to be biologically normal and has been maintained for over 5 years.

2.7.2 Detection of Heat Shock Protein 70-Enhanced Green Fluorescent Protein Expression

Cells expressing the reporter gene *hsp70*-eGFP will contain eGFP. As the GFP accumulates, the cells will fluoresce green when exposed to blue light. Expression of the *hsp70*-eGFP reporter gene was detected by viewing living transgenic embryos/larvae under a photomicroscope with a Nikon GFP-B filter cube. A Nikon Eclipse E600 equipped with a Nikon Y-FL Epifluorescence attachment was used.

2.8 Experimental Design

2.8.1 96 hr Acute Exposures and Calculation of Median Lethal and Median Effects Concentrations

Continuous 96 hr acute toxicity tests were first performed with wild-type larvae collected and maintained as described in section 2.1. Larval exposures began on the third day of development (72 hpf) when all of the chorions were shed and was continued until the eighth day of development (168 hpf) prior to initiation of feeding. Concentrations used for these exposures were 0 μ M (control), 10 μ M, 100 μ M, 300 μ M, 500 μ M, 700 μ M 1000 μ M, 2000 μ M, and 3000 μ M for AsIII, and 0 μ M (control), 10 μ M, 100 μ M, 500 μ M, 700 μ M 1000 μ M, 1500 μ M, 2500 μ M, 5000 μ M and 7500 μ M for AsV. Three replicate treatments (3 x 20 larvae) were exposed to each concentration. Solutions were changed twice daily during the 96 hr time frame and effects were assessed and recorded at these times. Gross effects included disorientation, erratic swimming and loss of equilibrium, edema, trunk abnormalities, immobility, necrosis, and mortality. Mortality, immobility, edema and trunk abnormalities were the least subjective and were therefore chosen as endpoints for establishing the dose-

response curve. At the conclusion of the 96 hr exposure the average number of larvae exhibiting one or more of these endpoints was calculated along with the standard error of the mean (SEM) and a dose-response curve was generated.

The median lethal (LC_{50}) and effective concentrations (EC_{50}) for AsIII and AsV were calculated from the dose-response curve established using the four endpoints observed during the 96 hr acute exposure: mortality, edema, trunk abnormalities, and immobility. However, the sigmoidal dose-response curve generated from this data must be converted to a linear format in order to predict a reliable LC_{50} and EC_{50} . Using procedures described in Gad (1999), a log-Probit transformation and linear regression of the data was performed from which the LC_{50} and EC_{50} along with their 95% confidence intervals were calculated. A table of Probit values (Gad 1999), was used to convert percent response to Probits and \log_{10} of the concentration was calculated using Microsoft Excel LOG function.

A log-Probit transformation results in a linearized format of the dose-response curve. Concentration values were converted to the log base 10 (\log_{10}) and the percent response converted to a probability unit (Probit). The Probit is an abstract unit that represents standard deviations from the mean of a normal distribution. Units of deviation from the mean are first converted to normal equivalent deviations (NED). The NED for 50% = 0. To avoid negative values 5 is added to the NED and therefore a 50% response (LC_{50} or EC_{50}) becomes a Probit of 5. Concentrations at which 100% and 0% response were observed cannot be transformed to Probits as this would correspond to plus and minus infinity of the normal distribution. Therefore, these were not used in the calculation of the EC_{50} and LC_{50} .

Transformation of the data resulted in a linearized plot of between 4-6 points with Log_{10} (concentration) on the x-axis and Probit on the y-axis. The coefficient of correlation was determined for the two variables and simple linear regression was performed using Microsoft Excel 9.0. The linear regression generates a regression equation from which the LC_{50} and EC_{50} along with 95% confidence intervals can be calculated.

Results from the 96 hr acute exposure studies were also used to determine the concentrations used in treatments for gene expression evaluation and comparison in wild-type and transgenic strains. Continuous 96 hr acute exposures were also performed using transgenic zebrafish exposed to Giant Gold Mine effluent discharge and with reference water samples from Giant Mine Baker Creek Outflow.

2.8.2 Acute Pulse Exposures for Wild-type and Transgenic Larvae

Endogenous *hsp70* expression in wild-type zebrafish and *hsp70*-eGFP expression in transgenic zebrafish were investigated and compared after exposure to AsIII and AsV. Using northern blot analysis of extracts from heat shocked zebrafish embryos, Lele *et al.* (1997) determined that peak *hsp70* mRNA signal is reached approximately 3 hours after the start of exposure. Therefore, in order to detect a strong mRNA signal wild-type zebrafish larvae were exposed to the various concentrations of AsIII and AsV for 3 hr beginning at 80 hpf and immediately fixed for *in situ* hybridization. Concentrations for As exposures were based on results from continuous (96 hr) exposures described above and were chosen to represent a low or no observed adverse effects concentration (100 μM for AsIII and AsV), a concentration at or about the EC_{50} (700 μM for AsIII; 1500 μM for AsV), and a high concentration that resulted

in 100% mortality (2000 μM AsIII; 7500 μM AsV). The positive control for *hsp70* expression consisted of a 1 hr heat shock (37°C) treatment group and the negative control consisted of exposure to system water only. Immediately following the 3 hr exposure, wild-type larvae were fixed for *in situ* hybridization after which tissues expressing *hsp70* mRNA were observed and counted in the whole larvae or in serial sections (process described in section 2.3). Transgenic strains were allowed to recover in clean system water for 24 hr to allow the GFP to accumulate, before live larvae were observed for *hsp70*-eGFP expression and expressing tissues were counted.

Expression of *hsp70*-eGFP was also investigated in transgenic zebrafish following an acute exposure to gold mine effluent discharge from Giant Gold Mine in the Northwest Territories. Concurrent exposures were carried out with reference waters from Giant Mine Baker Creek outflow. Both the effluent and outflow samples were applied to the larvae undiluted. After the 3 hr exposure the transgenic fish were allowed to recover in fresh water for 24 hr and live larvae were observed for *hsp70*-eGFP expression.

2.9 Statistical Analysis of Data

Differences in expression of *hsp70* and *hsp70*-eGFP between control and exposed larvae were tested for significance using Fisher's Exact test as described by Gad (1999) and Zar (1999). Fisher's Exact Test uses categorical data and is comparable to the two-way chi-square test. However, unlike chi-square analysis Fisher's Exact Test can be used with small sample sizes ($n < 50$) and allows for outcomes as low as zero to be included in calculations. These considerations were important when analyzing the

data in this study as sample sizes were all below 45 and there was no tissue expression in control larvae or in larvae exposed to low concentrations of As (ie. outcome= 0).

In this study the proportion of wild-type and transgenic larvae expressing *hsp70* and *hsp70*-eGFP in a given tissue were calculated for each treatment concentration and the results were arranged into a 2X2 contingency table. Fisher's Exact Test was used to compare differences in tissue expression between control and treatment groups as well as between AsIII and AsV treatment groups. An exact probability of significant difference was then calculated using Microsoft Excel 9.0.

3.0 Results

3.1 Acute Toxicity of Arsenite and Arsenate to Zebrafish Larvae

An acute (96 hr) continuous exposure was used to establish the dose-response relationship of AsIII and AsV in early life stage zebrafish. Larvae were continuously exposed to AsIII or AsV beginning at 72 hpf and ending at 168 hpf. Concentrations ranged from 0-3000 μM for AsIII, and 0-7500 μM for AsV. Gross effects observed in zebrafish larvae following exposure to either AsIII or AsV included edema, immobility, trunk abnormalities, loss of equilibrium, erratic swimming, necrosis and mortality (see Appendix 1 and Appendix 2 for raw data). Edema, defined as abnormal swelling, was observed mainly in the pericardial and thoracic regions. Trunk abnormalities mainly took the form of scoliosis (a curvature in the anterior-posterior body axis as viewed from a lateral position). Larvae exhibiting trunk abnormalities (C,D) or edema (E,F) are depicted in Figure 3.1. Larvae were considered immobile if they were lying on their side and failed to respond to tactile stimulation. Immobility was also accompanied by a slower heart rate. Absence of a heartbeat and observable gray necrotic tissue clearly defined mortality.

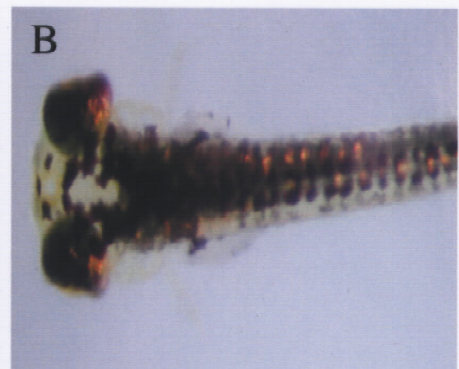
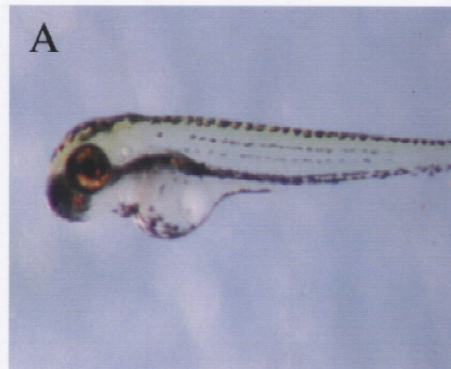
Edema, immobility, trunk abnormality, and mortality were used as endpoints to establish the dose-response relationship and for calculation of the LC_{50} and EC_{50} as they were the least subjective. The LC_{50} and EC_{50} along with 95% confidence intervals for AsIII were calculated to be: $\text{LC}_{50} = 772 \mu\text{M}$ (672 - 887 μM) and $\text{EC}_{50} = 570 \mu\text{M}$ (498 - 651 μM); and for AsV, $\text{LC}_{50} = 1347 \mu\text{M}$ (1043 - 1740 μM) and $\text{EC}_{50} = 1172 \mu\text{M}$

Figure 3.1 Gross effects in zebrafish larvae following continuous exposure to arsenic. Starting at 72 hpf, larvae were continuously exposed to AsIII concentrations ranging from 0-3000 μ M for 96 hr. Acute 96 hr exposure resulted in trunk abnormalities (C,D,) and cardiac edema (E,F). Other effects (not shown) included immobility, necrosis, and mortality.

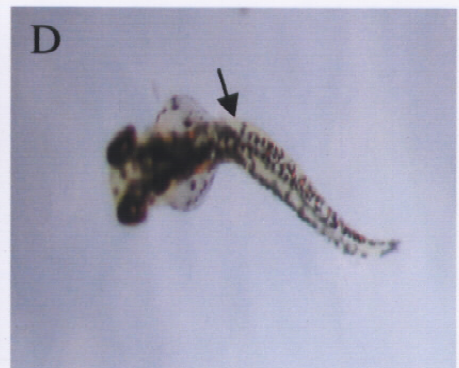
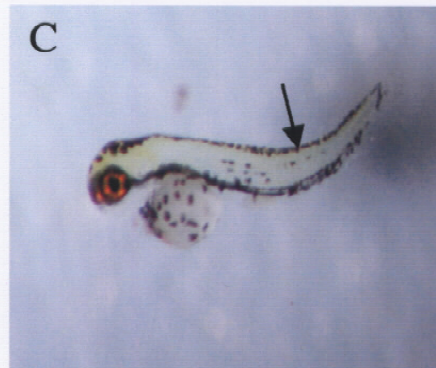
lateral

dorsal

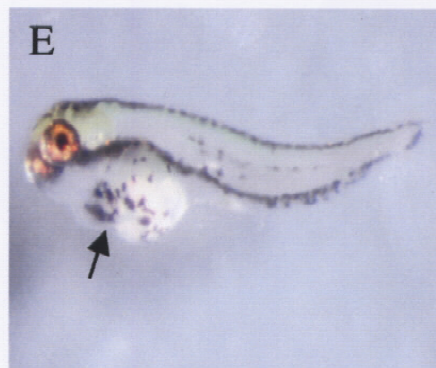
control



abnormal
trunk



edema



(842 - 1632 μ M). Graphs representing the percent response for mortality and for all endpoints are presented in Figures 3.2 and 3.3 along with the linear regression of their log-Probit transformation.

3.2 Expression Patterns of Heat Shock Protein 70 and Heat Shock Protein 70 – Enhanced Green Fluorescent Protein Under Non-Stressed Conditions and Following Heat Shock

Previous work in this lab determined expression patterns of *hsp70* and *hsp70*-eGFP in developing zebrafish larvae. Constitutive expression was observed in the lens of the eye between 24-42 hpf (*hsp70*) and after 48 hpf (*hsp70*-eGFP) and was not observed in any other tissues over the first 96 hours of development. Expression patterns at 80hpf were re-examined and confirmed for the present study. No expression of endogenous *hsp70* was observed under non-stressed conditions, whereas a 1 hr heat shock at 37°C resulted in upregulation throughout the larvae (Figure 3.4A,C). Lens expression of *hsp70*-eGFP in transgenic larvae was observed at 80 hpf (Figure 3.4B). Induction of *hsp70*-eGFP was also upregulated throughout the larvae in response to a 1 hr heat shock (Figure 3.4D).

3.3 Induction of Heat Shock Protein 70 and Heat Shock Protein 70-Enhanced Green Fluorescent Protein by Arsenic

Acute three hour exposures to As were carried out with wild-type and transgenic larvae using low, mid, and high concentrations of AsIII or AsV. Wild-type *hsp70* gene induction was investigated via *in situ* hybridization analysis using a DIG-labeled antisense *hsp70* RNA probe, and expression of the reporter *hsp70*-eGFP was examined in the living transgenic larvae.

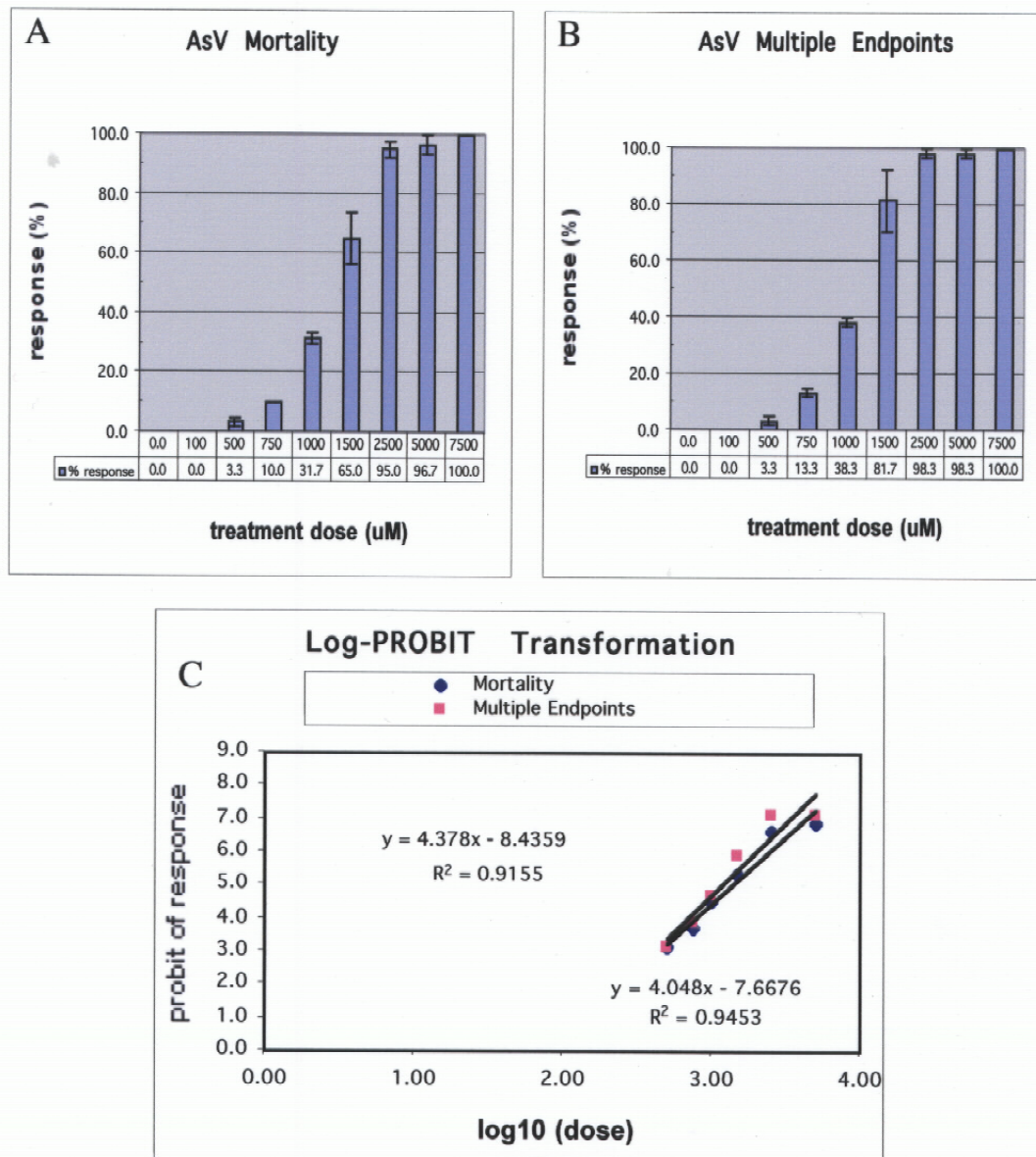


Figure 3.2 Dose-response relationship for arsenate toxicity to zebrafish larvae. Zebrafish larvae were continuously exposed to AsV dissolved in system water for 96 hours beginning at 72 hpf and ending at 168 hpf. The proportion of larvae per treatment concentration exhibiting mortality or multiple effects are shown in panels A and B respectively. Each bar is the average of three replicate treatments expressed as a percent shown with error bars. Panel C shows the log-Probit transformations of the mortality and multiple endpoints data with the best fit line predicted for the linear regression and the coefficient of determination (R^2). Multiple endpoints and mortality regression equation with coefficient of determination in upper left corner and lower right corner respectively.

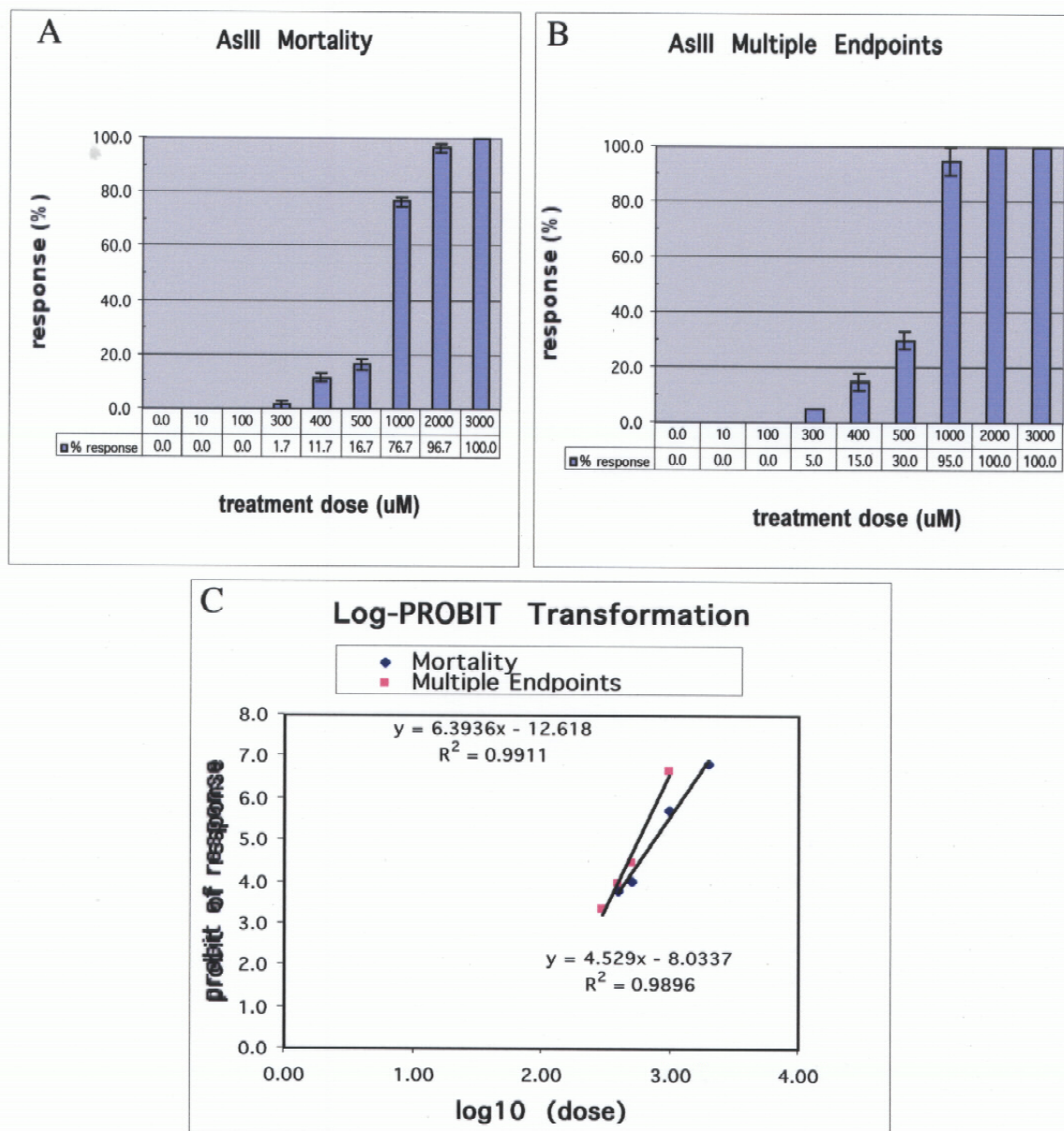


Figure 3.3 Dose-response relationship for arsenite toxicity to zebrafish larvae. Zebrafish larvae were continuously exposed to AsIII dissolved in system water for 96 hours beginning at 72 hpf and ending at 168 hpf. The proportion of larvae per treatment concentration exhibiting mortality or multiple effects are shown in panels A and B respectively. Each bar is the average of three replicate treatments expressed as a percent shown with error bars. Panel C shows the log-Probit transformations of the mortality and multiple endpoints data with the best fit line predicted for the linear regression and the coefficient of determination (R^2). Multiple endpoints and mortality regression equation with coefficient of determination in upper left corner and lower right corner respectively.

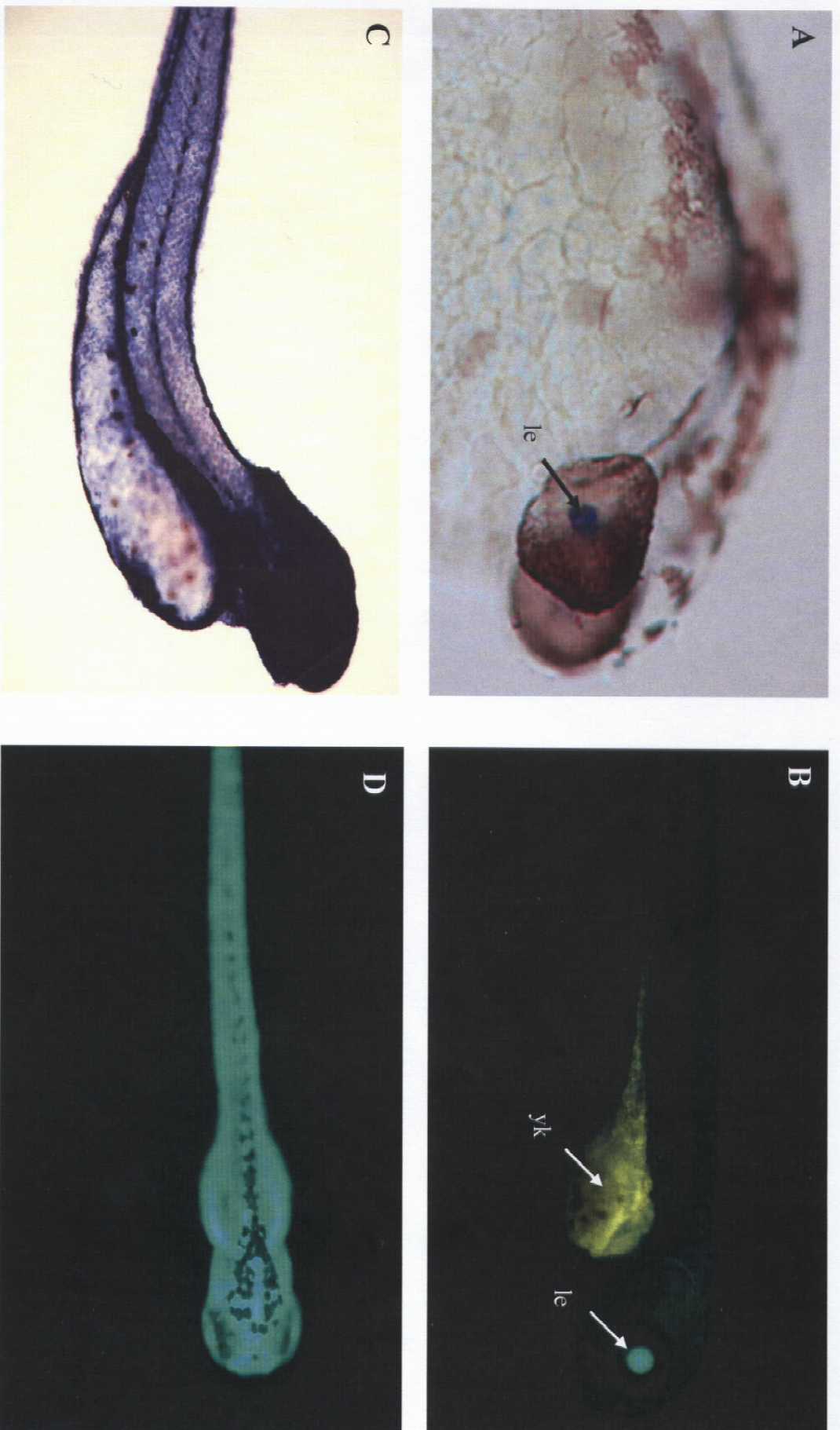


Figure 3.4 Expression patterns of endogenous *hsp70* and *hsp70-eGFP* under control and heat shock conditions. Unstressed wild-type larvae express *hsp70* in the developing lens between 24-48hpf (A). Constitutive lens expression is still evident in transgenic larvae at 80hpf, and background autofluorescence in the yolk and digestive tube can be seen in all transgenic larvae including controls (B). Heat shock results in overall induction of *hsp70* (C) and *hsp70-eGFP* expression throughout the larvae (D). yk, yolk; le, lens.

3.3.1 Arsenite-Induced Expression

Following exposure to AsIII, *hsp70* expression was detected in the liver, gills, GIT (any epithelium along the gastrointestinal tract including gut and mouth epithelium), pericardial muscle, olfactory rosette and the skin. Whole mount photos (Figure 3.5) show a dose-dependent increase in *hsp70* in the epithelium, liver and GIT (as determined by percent of larvae expressing *hsp70* mRNA in target tissues). A dose-dependent increase in the number of tissue types expressing was also observed. Serial sections of larvae exposed to 700 μ M AsIII revealed expression in the skin, liver, and GIT (not shown). Exposure to 1000 μ M or 2000 μ M AsIII resulted in expression in the skin, gills, liver, pericardial muscle and GIT (Figure 3.6). Morphological integrity did not always allow definitive determination of expression in the olfactory region of wild-type larvae; therefore, expression in this tissue was not used in subsequent calculations. To determine if the reporter gene *hsp70*-eGFP accurately mimicked endogenous *hsp70* expression, transgenic larvae were exposed to AsIII at concentrations analogous to those used in wild-type exposures. Expression patterns of *hsp70*-eGFP detected in transgenic larvae 24 hr after exposure to AsIII were similar to expression patterns detected in wild-type larvae (Table 3.1). Transgenic larvae exposed to 700 μ M AsIII revealed reporter gene induction in skin (not shown), liver and GIT (Figure 3.7). Expression of *hsp70*-eGFP was observed in the skin, gills, liver, GIT, and cardiac region following exposure to 1000 μ M AsIII and in the skin, gills, liver, GIT, cardiac region, and olfactory epithelium following exposure to 2000 μ M (Figure 3.7). No expression was observed in tissues other than the lens in control larvae.

Figure 3.5 Dose-dependent expression of endogenous *hsp70* in wild-type larvae following a 3 hour arsenite exposure and *in situ* hybridization. No expression is evident in control larvae (A). Expression of *hsp70* is evident only in the liver in whole mount larvae following exposure to 700 μ M AsIII (B); gill and head epithelium, liver, and GIT following exposure to 1000 μ M AsIII; and head, trunk and tail epithelium, gill epithelium, liver, and GIT following exposure to 2000 μ M AsIII. Arrows indicate areas of expression. ep, skin epithelium; GIT, gastorintestinal tract; gi, gill epithelium; li, liver.

control



700 μM



1000 μM



2000 μM

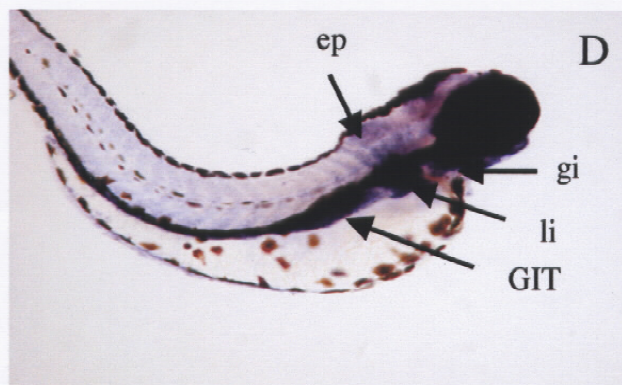


Figure 3.6 Tissue specific expression of endogenous hsp70 as a result of exposure to arsenite and detected with *in situ* hybridization to an antisense riboprobe. Serial sections of zebrafish larvae exposed to 2000 μ M arsenite reveal expression of *hsp70* in skin and digestive tract epithelium (B,F, and H), gills (D), pericardial muscle (not shown), and liver (H). No expression is seen in control larvae (A,C, E, and G). ep, skin epithelium; GIT, gastrointestinal tract epithelium; gi, gill; li, liver.

control

2000 μ M

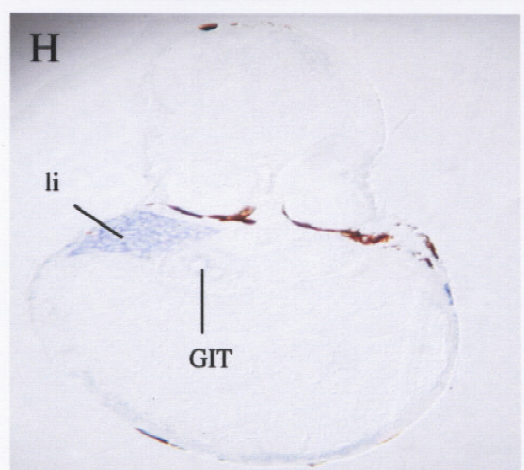
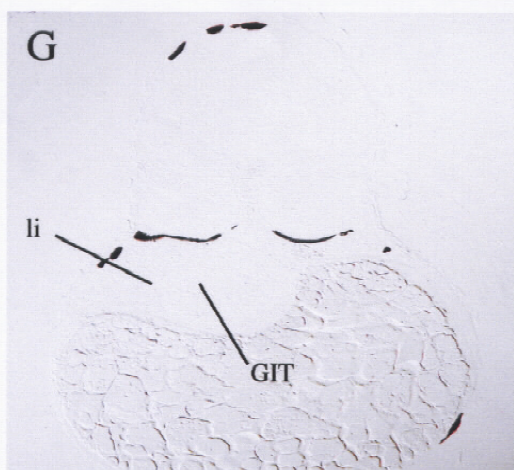
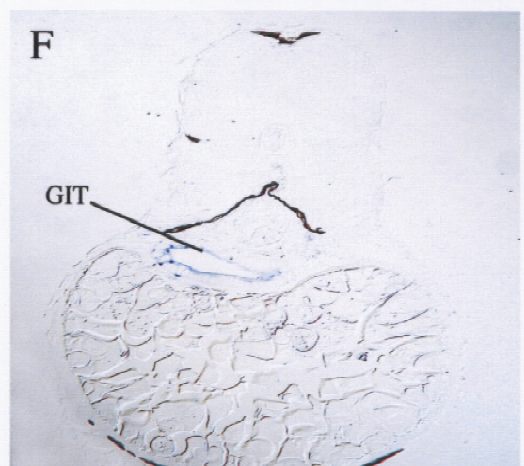
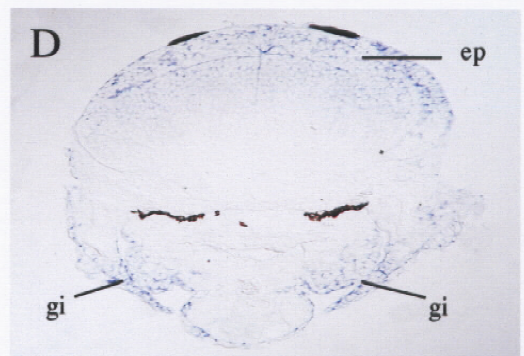
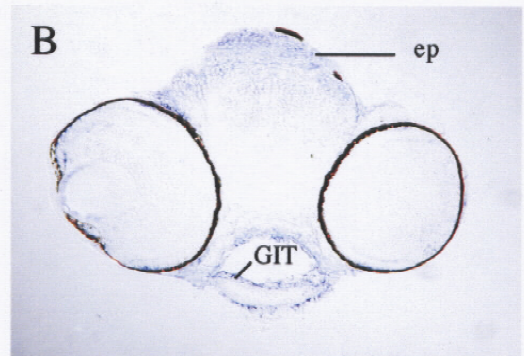
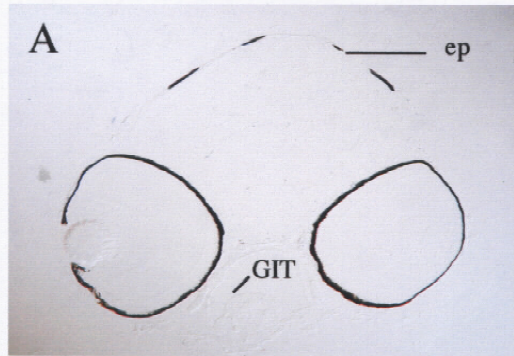


Table 3.1 Tissues expressing *hsp70* or *hsp70*-eGFP following arsenite exposure

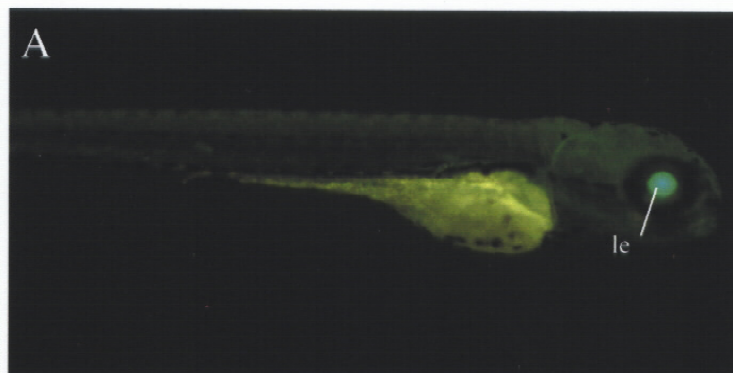
Treatment		% of Embryos Expressing in Tissue			
fish and gene type	concentration (μM)	gills/skin	cardiac	liver	GIT
wild-type <i>(hsp70)</i> n=45	0	0	0	0	0
	700	8.9*	0*	15.6	20
	1000	66.7	40	46.7	60
	2000	100	93.3	60	100
transgenic <i>(hsp70-eGFP)</i> n=15	0	0	0	0	0
	700	33*	0	46.7	46.7
	1000	71.4	57.1	78.6	85.7
	2000	100	78.6	85.7	92.9

Tissues expressing were counted and expressed as a percent for each concentration treatment. Serial sections of fixed wild-type larvae were used to count tissues expressing *hsp70*. Tissues expressing *hsp70*-eGFP were viewed and counted in live transgenic larvae. Fisher's Exact test was used to calculate the probability of no difference in expression of *hsp70* ($p < 0.012$) or *hsp70*-eGFP ($p < 0.025$) in tissues of exposed and untreated larvae. The extremely low p-values indicated a significant difference between exposed and untreated larvae except where indicated.

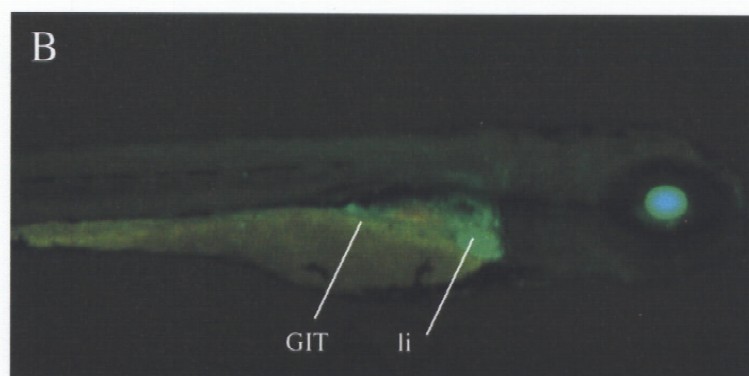
* difference not statistically significant ($p > 0.05$)

Figure 3.7 Dose-dependent induction of *hsp70*-eGFP by arsenite in transgenic larvae. Larvae (80 hpf) were exposed to concentrations of AsIII ranging from 0-2000 μ M for 3 hr and then allowed to recover for 24 hr in fresh water. Expression in the lens is constitutive and is seen in all larvae (A-E) including controls (A) (same control as used in figure 3.4). Following exposure to 2000 μ M AsIII *hsp70*-eGFP is expressed in olfactory epithelium (E), skin, GIT, liver, and cardiac region (D). Expression is seen in the liver, gut, and skin at 1000 μ M (C) and only in the liver and GIT at 700 μ M (B). Background autofluorescence can be seen in the yolk region of some larvae (A). le, lens; ep; skin epithelium; GIT, gastrointestinal epithelium; li, liver; ca, cardiac region.

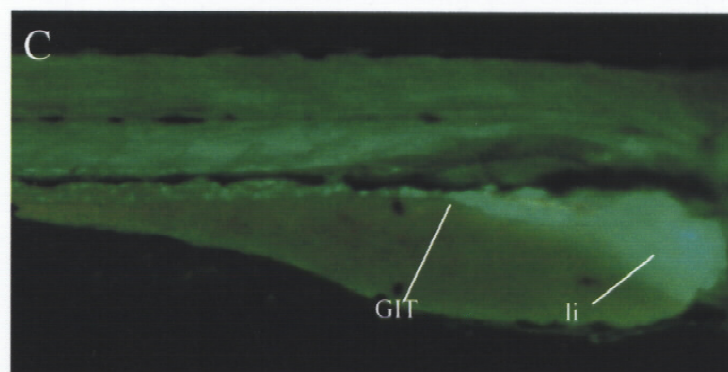
control



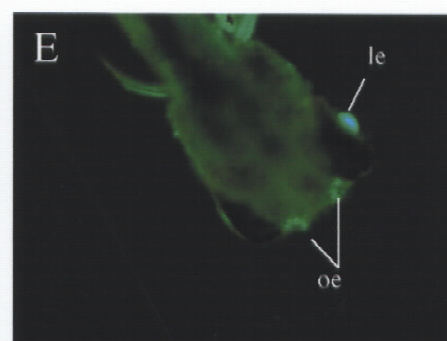
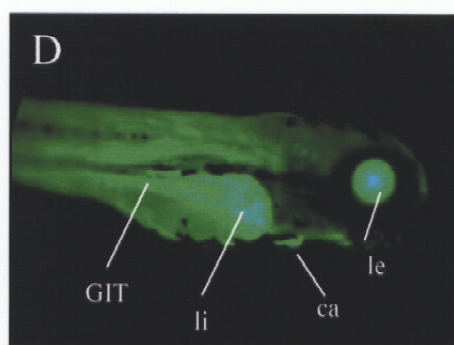
700 μ M



1000 μ M



2000 μ M



The proportion of larvae per treatment expressing *hsp70* (wild-type) or *hsp70*-eGFP (transgenic) in a given tissue were calculated from counts of the serial sections and in live transgenic larvae, respectively (Table 3.1). Differences between larvae exposed and control larvae expressing *hsp70* in a given tissue were tested for significance using Fisher's exact test (Appendix 3). Differences in expression of *hsp70* in a given tissue were highly significant for all tissue types ($p < 7.2E-07$) between control larvae and larvae exposed to 1000 μM or 2000 μM AsIII. Differences in tissue expression between untreated larvae and those exposed to 700 μM AsIII were significant ($p < 0.012$) except in the skin ($p > 0.05$). The proportion of transgenic larvae expressing *hsp70*-eGFP in a given tissue was significantly different for all tissues ($p < 0.0063$) between control larvae and larvae exposed to 1000 μM or 2000 μM AsIII. There was also a significant difference ($p < 0.023$) in expression of *hsp70*-eGFP in a given tissue between untreated and larvae exposed to 700 μM AsIII except in the skin ($p > 0.05$).

3.3.2 Arsenate-Induced Expression

Induction of *hsp70* and *hsp70*-eGFP following a three hour exposure to AsV was investigated in wild-type and transgenic larvae. Whole-mount photos of wild-type larvae (Figure 3.8), show a dose-dependent increase in *hsp70* mRNA transcripts as determined by percent of larvae expressing *hsp70* mRNA in a given target tissue. Serial sections confirm the dose-dependent increase in tissues expressing *hsp70*. Transcripts were detected in the liver, GIT, skin, pericardial muscle, and gill area in larvae exposed to 7500 μM AsV (Figure 3.9). Exposure to 2500 μM AsV resulted in expression in skin, gills, liver, pericardial muscle, and GIT while exposure to 1500 μM

Figure 3.8 Dose-dependent expression of endogenous *hsp70* in wild-type larvae following a three hour arsenate exposure and *in situ* hybridization. Larvae (80 hpf) were exposed to AsV for three hours and immediately fixed for *in situ* hybridization. Expression of *hsp70* is evident only in the liver and GIT following exposure to 1500 μ M AsV; head epithelium, gill area, liver, and GIT express *hsp70* following exposure to 2500 μ M AsV; and expression is seen in the head and trunk epithelium, gill, liver and GIT after exposure to 7500 μ M AsV. No expression is evident in control larvae (A). Arrows indicate areas of expression. ep, skin epithelium; gi, gill; GIT, gastrointestinal tract epithelium; li, liver.

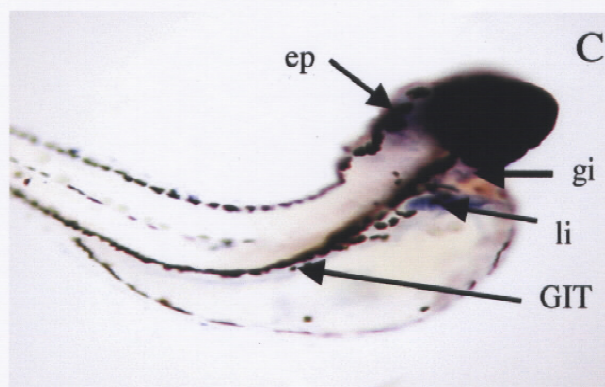
control



1500 μM



2500 μM



7500 μM

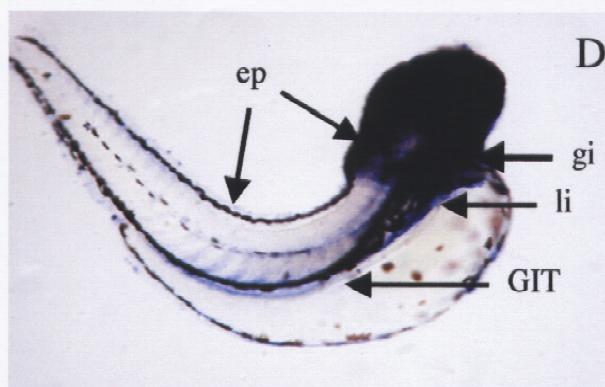
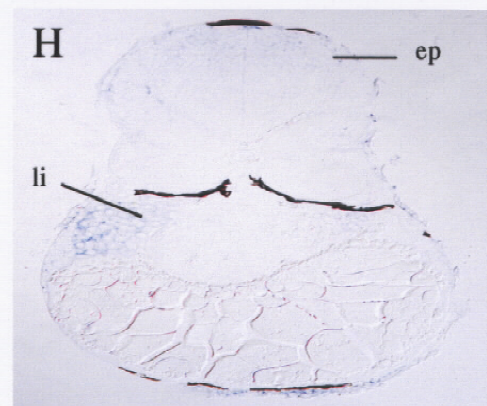
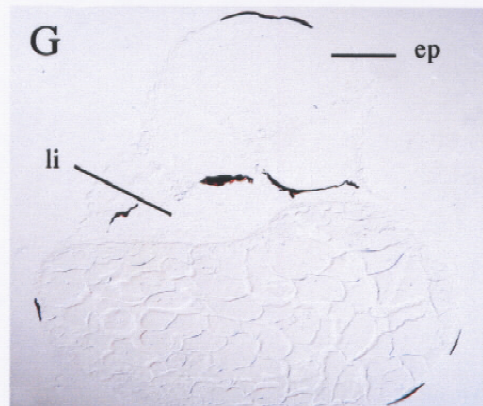
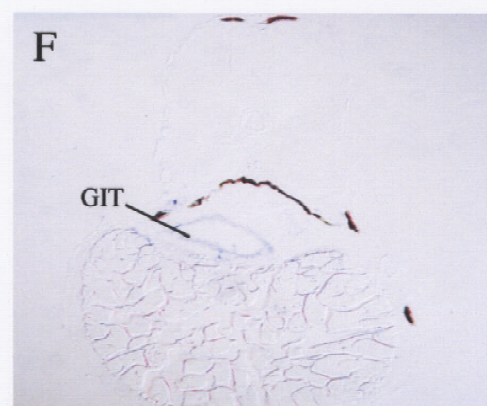
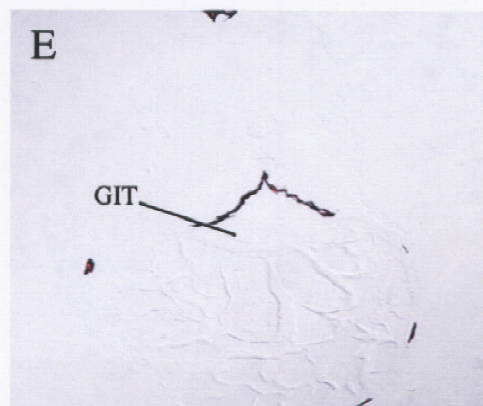
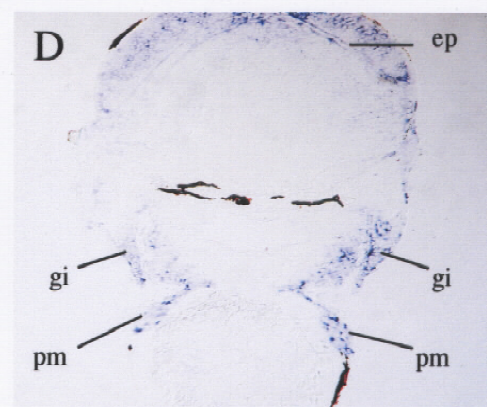
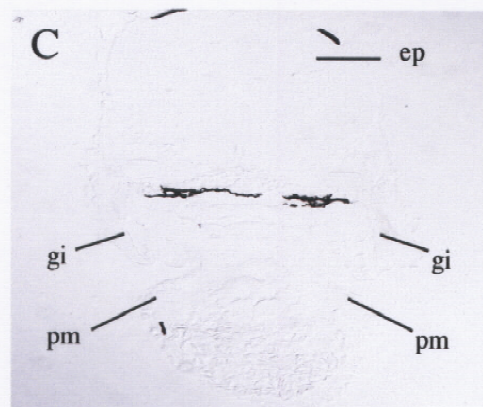
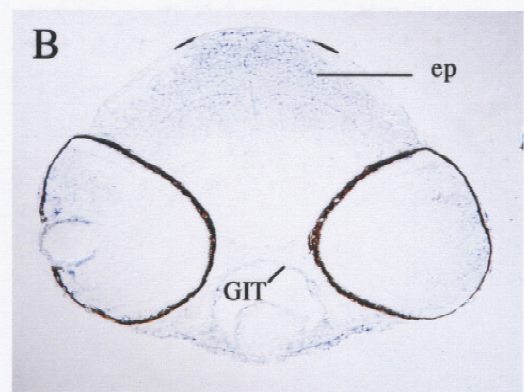
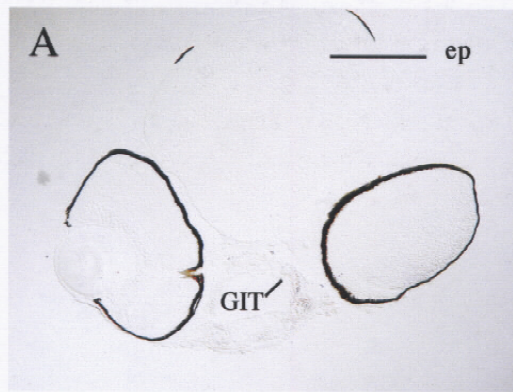


Figure 3.9 Tissue specific expression of endogenous *hsp70* as a result of exposure to arsenate and detected with *in situ* hybridization. Larvae (80 hpf) were exposed to AsV for 3 hours and immediately fixed for *in situ* hybridization. Serial sections of zebrafish larvae exposed to 7500 μ M arsenate reveal expression of *hsp70* in skin and GIT epithelium (B and F), gills and pericardial muscle (D), and liver (H). No expression is seen in control larvae (A, C, E, G). ep; skin epithelium; GIT, gastrointestinal tract epithelium; gi, gill; li, liver; pm, pericardial muscle.

control

7500 μ M

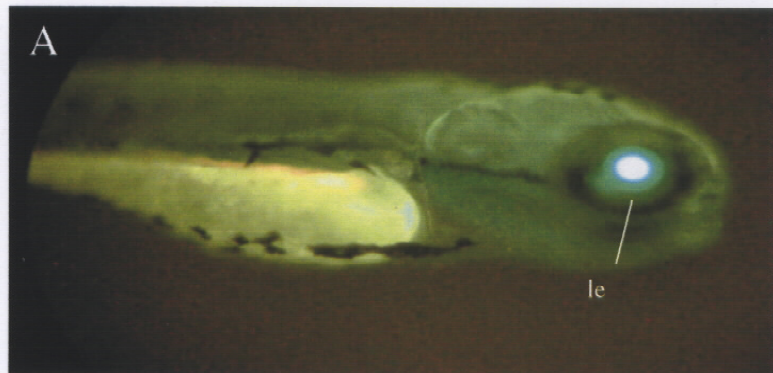


resulted in expression in the skin, liver, and GIT. Examination of larvae exposed to 700 μM and 0 μM (control) AsV indicated no induction of *hsp70*.

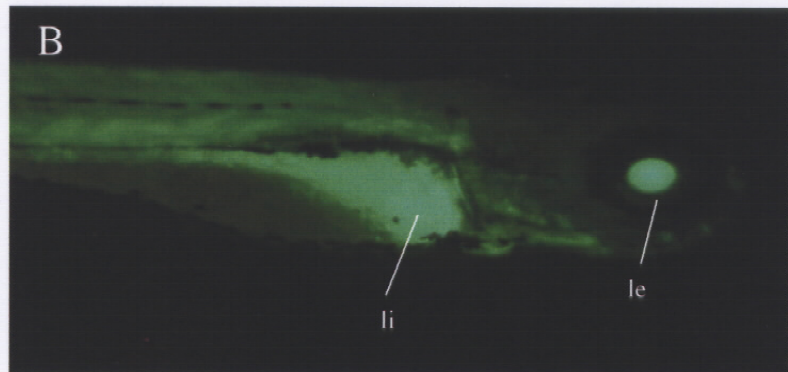
Expression patterns of *hsp70*-eGFP detected in transgenic larvae 24 hr after exposure to AsV were similar to expression patterns for *hsp70* detected in wild-type larvae. Observation of whole living transgenic larvae revealed a dose-dependent induction of the reporter gene *hsp70*-eGFP (Figure 3.10), as determined by percent of larvae expressing *hsp70*-eGFP in a given tissue. A three hour exposure to 7500 μM and 2500 μM AsV resulted in expression in the skin, cardiac region, liver, and GIT. Exposure to 1500 μM resulted in expression in the liver, GIT, and skin. No expression was seen at lower concentrations. As with larvae exposed to AsIII, the proportion of larvae per concentration found to express *hsp70* (wild-type) or *hsp70*-eGFP (transgenic) in a given tissue following AsV exposure was calculated and expressed as a percent (Table 3.2). Differences between the control and exposed larvae expressing *hsp70* in a given tissue were significant for all tissue types ($p < 0.002$) at all concentrations except the pericardial muscle ($p > 0.05$) following exposure to 1500 μM AsV. Differences between controls and exposed transgenic larvae expressing *hsp70*-eGFP in a given tissue were significant for all tissues following exposure to 2500 μM and 7500 μM AsV ($p < 0.025$). Following exposure to 700 μM , there was a significant difference in the proportion of exposed larvae expressing *hsp70*-eGFP in the liver compared to control larvae ($p < 0.025$), however, there was no significant difference in remaining tissues ($p > 0.05$).

Figure 3.10 Dose-dependent induction of *hsp70*-eGFP by arsenate in transgenic larvae. Larvae (80 hpf) were exposed to concentrations of AsV ranging from 0-7500 μ M for three hours and then allowed to recover for 24 hr in fresh water. Expression in the lens is constitutive and is seen in all larvae including controls (A-E). Following exposure to 7500 μ M AsIII *hsp70*-eGFP is expressed in olfactory epithelium (not shown), skin, GIT, liver, and cardiac region (E,F). Expression is seen in the liver, GIT, and skin at 2500 μ M (C,D) and only in the liver at 1500 μ M (B). Background autofluorescence can be seen in the yolk region of some larvae (A). le, lens; ep; skin epithelium; GIT, gastrointestinal tract epithelium; li, liver; ca, cardiac region.

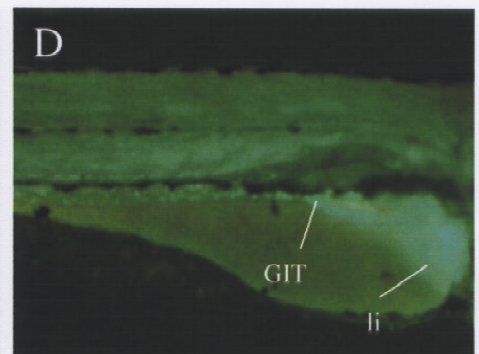
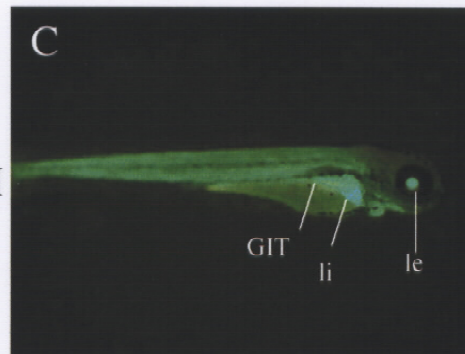
control



1500 μ M



2500 μ M



7500 μ M

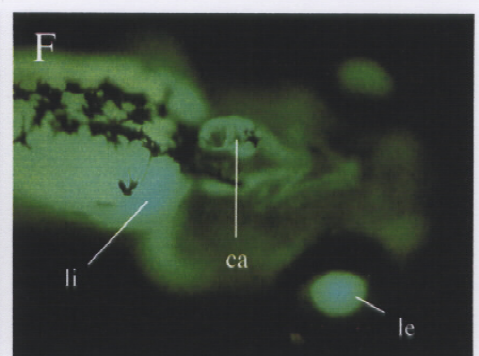
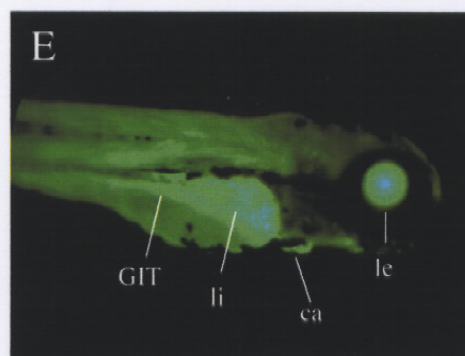


Table 3.2 Tissues expressing *hsp70* and *hsp70*-eGFP following arsenate exposure

Treatment		% of Embryos Expressing in Tissue			
fish and gene type	concentration (μM)	gills/skin	cardiac	liver	GIT
wild-type <i>(hsp70)</i> n=45	0	0	0	0	0
	1500	21	9.3*	30	30
	2500	60	51.1	42.2	48.9
	7500	100	82.2	55.6	66.7
transgenic <i>(hsp70-eGFP)</i> n=15	0	0	0	0	0
	1500	20*	0	46.7	33*
	2500	66.7	46.7	60	60
	7500	100	78.6	92.9	100

Tissues expressing were counted and expressed as a percent for each concentration treatment. Serial sections of fixed wild-type larvae were used to count tissues expressing *hsp70*. Tissues expressing *hsp70*-eGFP were viewed and counted in live transgenic larvae. Fisher's Exact test was used to calculate the probability of no difference in expression of *hsp70* ($p < 0.002$) or *hsp70*-eGFP ($p < 0.025$) in tissues of exposed and untreated larvae. The extremely low p-values indicates a significant difference between exposed and untreated larvae except where indicated.

* difference not statistically different ($p > 0.05$)

3.4 Heat Shock Protein 70-Enhanced Green Fluorescent Protein Induction

Following Exposure to Mine Effluent Discharge

Samples of effluent discharge were obtained from the Giant Gold Mine in the Northwest Territories along with water from Giant Mine Baker Creek outflow, and provided by Dr. Karsten Liber. Transgenic zebrafish larvae were exposed to either the effluent discharge or the water from Baker Creek following the same procedures used in the 96 hr As exposures (section 2.9.1). The 96 hr acute exposures resulted in no observable adverse effects and no observable induction of *hsp70*-eGFP. Analysis of the effluent discharge and Baker Creek samples by the Experimental Mineralogy-Geochemistry Laboratory at the University of Saskatchewan determined As levels of 406 ppb (5 μ M) and 114 ppb (1 μ M) respectively. Levels of other potential toxicants present in the samples were correspondingly low (Table 3.3).

Table 3.3 Concentration of metals detected in Giant Mine effluent discharge and Baker Creek outflow.

	Element Detected and Concentration (ppb)								
	As	Cd	Hg	Pb	Ni	Fe	Cu	Al	U
Bakers Creek Outflow	114.2	1.3	ud	0.1	1.9	118.2	8.4	17.8	0.4
Giant Mine Effluent Discharge	405.8	1.3	ud	0.2	14.6	415.2	11.4	99.4	1.5

Concentrations of potentially toxic metals in Giant Mine effluent discharge and reference waters as analyzed by the Experimental Mineralogy Geochemistry Laboratory at the University of Saskatchewan. As, arsenic; Cd, cadmium; Hg, mercury; Pb, lead, Ni, nickel; Fe, iron; Cu, copper; Al, aluminum; U, uranium; ud, undetected.

4.0 Discussion

The objective of this study was to evaluate *hsp70* expression as an indicator of As exposure, and to examine the accuracy of the *hsp70*-eGFP reporter gene construct as an indicator of endogenous *hsp70* expression. It was hypothesized that *hsp70* is expressed in target tissues of As exposure and could act as a biomarker of As cellular toxicity. It was also hypothesized that *hsp70*-eGFP expression in transgenic zebrafish would closely mimic endogenous *hsp70* expression and could therefore be used as an accurate *in vivo* assay method. To this end, endogenous *hsp70* and *hsp70*-eGFP expression patterns were examined and compared in wild-type and transgenic larvae following exposure to AsIII and AsV. As well, expression of *hsp70*-eGFP was examined in transgenic larvae following exposure to effluent discharge from Giant Gold Mine. Conclusions and a discussion of the results are included below.

4.1 Conclusions

The primary objectives of this study were (a) to determine if *hsp70* is a reliable indicator of exposure to As and (b) if the transgenic zebrafish model would prove to be a rapid and accurate *in vivo* assay method indicating toxic exposure to As. In regards to this a number of conclusions were reached:

1. AsIII and AsV induce *hsp70* in a tissue-specific and dose-dependent manner.

To establish the validity of the transgenic zebrafish model for assessing As toxicity it was first necessary to determine expression patterns of *hsp70* in wild-type

zebrafish in response to AsIII and AsV exposures. To establish expression patterns of *hsp70* in wild-type larvae exposed to As, three-hour exposures were carried out. Concentrations of AsIII or AsV representing low, mid-, and high concentrations (based on acute toxicity studies) were used and resulted in *hsp70* induction in specific tissues (epithelium, gills, GIT, liver, pericardial muscle and olfactory epithelium) that are potential targets of, or sensitive to, As exposure. Also, an increase in *hsp70* expression (as determined by percent of larvae expressing *hsp70* mRNA in target tissues) was observed in these tissues as the larvae were exposed to increasingly higher concentrations of As. The dose-dependency and tissue-specificity of *hsp70* induction in response to As exposure indicates that it is a reliable indicator of As exposure at high levels. These results, along with previous work done with Cd, suggest that *hsp70* is a biomarker of toxicity and further exposures to other toxicants would be useful in confirming this hypothesis.

2. Induction of the reporter gene *hsp70*-eGFP closely mimics endogenous *hsp70* induction in response to As exposure.

Exposure of transgenic larvae to As resulted in observable expression of *hsp70*-eGFP in tissues comparable to those expressing endogenous *hsp70* in the wild-type larvae exposed to As. Both endogenous *hsp70* and *hsp70*-eGFP were expressed in epithelium, gills, GIT, liver, and olfactory epithelium. These results suggest that the transgenic zebrafish model could act as a rapid and accurate *in vivo* assay method for As exposure. However, the high concentrations of As needed to induce *hsp70* or *hsp70*-eGFP indicate that this model may not be sensitive enough for environmental

monitoring but would be an excellent model system for developmental or mechanistic studies. The transgenic zebrafish model allows observation of affected cells in the living organism and the same fish can then be followed into later life stages. Thus, it may be a useful model to study possible abnormalities in later life stages resulting from early exposure.

3. AsIII is a stronger inducer of *hsp70* than AsV but notably high concentrations of both were needed to induce *hsp70*.

Induction of *hsp70* was observed following exposure to AsIII and AsV. However, in accordance with the greater toxicity of AsIII, exposure of larvae to higher concentrations of AsV was required for observable *hsp70* expression. Correspondingly, traditional measures of toxicity, such as the LC₅₀ and EC₅₀ were also higher for AsV. The LC₅₀ and EC₅₀ of AsV were determined to be 1.7X and 1.6X higher than that of AsIII. However, exposure to extremely high concentrations of AsIII or AsV was required for observable induction of *hsp70* or *hsp70*-eGFP suggesting that larvae do not accumulate As. Zebrafish, as well as a number of other species of fish, appear to be resistant to As toxicity. This may be due to inhibited uptake or enhanced excretion. Further studies on As accumulation and total body burden following As exposure would be helpful in elucidating this apparent resistance.

4. Exposure to water contaminated with tailings from the Giant Gold Mine did not result in any appreciable induction of *hsp70*-eGFP indicating a low acute toxicity.

Exposure to the Giant Mine effluent discharge for 96 hr resulted in little or no induction of *hsp70*-eGFP. Analysis of the contaminated water revealed a very low concentration (5.4 μ M) of As and other potential toxicants indicating that perhaps remediation efforts are successful.

4.2 Acute Toxicity of Arsenite and Arsenate

The toxicity of As can be altered by a number of factors and, because of this, there is a wide variation in the reported critical effects levels of As in fish. Direct comparison of reported values is complicated by variables such as developmental stage, exposure durations, and species differences, all of which can affect the LC₅₀. As well, water chemistry can influence the speciation and availability of As and can, therefore, also have an effect on the LC₅₀. It is pertinent to consider these factors before discussing the results of the present study. The properties and effects of As have been extensively studied and comprehensively reviewed in WHO (2001). Unless otherwise indicated the information below is based on this source.

4.2.1 Water Characteristics

Water chemistry is a principal consideration when discussing the toxicity of As. It can influence what species of As is predominantly present in an aqueous solution as well as the availability of the As species. Depending on the pH and the ionic environment, As can have a range of solubilities in water affecting bioavailability, and solubility can be a modifying factor of As toxicity particularly in natural environments. Water-soluble toxicants are more readily available to organisms than water-insoluble chemicals that are adsorbed or bound to biotic or abiotic components (Rand and Petrocelli, 1985). The presence of other constituents such as metal ions and organic

materials can affect As availability as co-precipitation and adsorption results in reduced concentrations of dissolved As in water. For example, high calcium concentrations in water can result in a relatively insoluble salt of calcium arsenite ($\text{Ca}(\text{AsO}_2)_2$) to form reducing the available AsIII. In natural environments, high concentrations of phosphate, bicarbonate, or silicate can increase the availability of As by competing for adsorption sites in certain types of soil. Conversely, in aqueous solutions, high concentrations of phosphates, with which AsV shares the same transport mechanism, can greatly reduce the uptake and toxicity of AsV (Gonzalez et al, 1995). As well, the solubility of some forms of As, such as arsenic trioxide, can be very low in purely aqueous solutions within biological pH ranges, and only changes significantly at extreme pH levels (Webb, 1966). However, most forms of As used in acute toxicity studies (e.g. sodium arsenite and sodium arsenate) in laboratory settings are considered highly soluble in water across the range of pH levels (5-8) commonly used.

Speciation can also affect the toxicity of As with trivalent species generally considered to be more toxic than pentavalent species. Speciation is complex and can be affected by a number of factors such as Eh, pH, and biological activities. In general, however, arsenate tends to be the predominant species present in well oxygenated water, while anaerobic conditions favor arsenite formation.

4.2.2 Animal Species Differences

In separate studies by Buhl and Hamilton (1990; 1991) inter- and intra-species differences have been demonstrated following exposure to a number of inorganics. Although no single species was consistently more sensitive to all toxicants tested, there were significant differences between comparable life-stages of Arctic grayling

(*Thymallus arcticus*) , coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*Oncorhynchus mykiss*) with some species demonstrating a greater sensitivity in general. Juvenile Alaska grayling were found to be at least 10 times more sensitive to copper, zinc, arsenate, and lead than were juvenile Alaska coho salmon (Buhl and Hamilton, 1990). In a similar study (Buhl and Hamilton, 1991), the alevin stage of Arctic grayling was determined to be more sensitive to 6 of 9 toxicants tested, including AsIII, than coho salmon and rainbow trout. Differences in LC₅₀'s ranged from 2.4 to 4.7 fold, depending on the inorganic tested. However, the juvenile stage of rainbow trout was found to be more sensitive to 5 of the 9 inorganics tested with differences in LC₅₀'s ranging from 1.2- to 5.6-fold. Juvenile rainbow trout were also found to be more sensitive than zebrafish to As in the form of arsenic trioxide in a study by Tisler and Zagorc-Koncan (2002); however, the life-stage of the zebrafish used was not stated and could have had an impact on the LC₅₀.

Considerable intra-species differences have also been demonstrated by Buhl and Hamilton (1990) where differences between 96hr LC₅₀'s for arctic grayling juveniles from Alaska and Montana were 1.2- to 1.5-fold for zinc, 5.6- to 10-fold for AsV, and 11.1- to 19.1-fold for copper. Juvenile coho salmon from Washington and Alaska also differed significantly in sensitivity to copper and lead but were equally tolerant to AsV. Although sensitivity differences vary with the toxicant used, both studies by Buhl and Hamilton (1990; 1991) demonstrate that Arctic grayling are the most sensitive of the three species to both AsIII and AsV. Factors such as size, variable test conditions, and differences in the chemical form of toxicant used may have some bearing on the

variable sensitivities, however, when test conditions are similar, differences in susceptibility are likely genetically based.

4.2.3 Developmental Stage and Exposure Duration

Although acute toxicity studies frequently report 96 hr LC₅₀'s, alternative exposure durations such as 24, 48, and 144 hours are also used. Lethal effects levels can vary considerably when comparing results from acute studies carried out over different exposure periods. Buhl and Hamilton (1990) established LC₅₀'s of 67.0 µM and 417.8 µM for Arctic grayling exposed to As for 96 and 24 hours respectively. Sorensen (1976) reported a 6.7 fold difference in median lethal effects levels for green sunfish (*Lepomis cyanellus*) exposed to AsV for 12 and 48 hours. During early developmental stages organisms undergo vast changes in relatively short time periods of time. Longer exposure durations are more likely to include stages of variable sensitivities as well as allowing more time for uptake, distribution, and ultimately, for critical concentrations to be reached at target sites.

Although embryogenesis involves a vast number of changes, the chorion acts as a protective barrier to many toxicants and therefore, this stage is often more tolerant than post-hatch stages to metals and other toxicants (Eaton *et al.*, 1978; McKim *et al.*, 1978; Shazili and Pasco, 1986). Post-hatch stages, however, lack a protective barrier and can vary substantially in susceptibility to chemicals. As well, the same terminology is often used for the various early life-stages of fish (i.e. alevin, fry, juvenile) although these stages may encompass different time frames for different species. For example, the period between hatching (alevin stage) and swim up fry for fathead minnows and brook trout are 2-3 days and 21-28 days respectively. Therefore, developmental stage

and exposure times used are important considerations when comparing reported critical effects levels in acute toxicity tests. The sensitivity of early life stages of fish to a variety of toxicants has been examined in a number of studies and results suggest that the juvenile stage of most species is, overall, the most sensitive post-hatch early life stage (Chapman, 1978; Van Leeuwen, 1985; Buhl and Hamilton, 1990; 1991). Juvenile and alevin stages were used by Buhl and Hamilton (1991) to examine the relative sensitivity of three species of fish (Arctic grayling, coho salmon, and rainbow trout) to nine inorganics. In 89% of the tests they performed, juveniles of each species were equally or more sensitive than alevins to the inorganics. Although alevins of some species were slightly more sensitive to some of the metals such as silver, the juvenile stage for all species tested were more sensitive to AsIII with LC₅₀'s of 13.7 mg/L (182.9 µM), 18.5 mg/L (247 µM), and 16.0 mg/L (213.6 µM) for Arctic grayling, coho salmon, and rainbow trout respectively. Median lethal effect concentrations for alevins were 27.7 mg As/L (369.7 µM), 49.4 mg As/L (659.4 µM), and 91.0 mg As/L (1214.6 µM). In an earlier study, Buhl and Hamilton (1990) demonstrated that alevins and swim-up fry of the same three species were also more tolerant than juveniles to AsV with LC₅₀'s between 5.1 mg/L (68.1 µM) – 67.5 mg/L (900.9 µM) for juveniles and 102 mg/L (1361.4 µM) - >360 mg/L (4805.1 µM) for alevins.

4.2.4 Feeding Effects

The effects of feeding must be taken into consideration when utilizing life-stages, such as juveniles, for acute 96 hr testing. Past the yolk-sac stage, an exogenous source of food is required and can become a factor in the apparent toxicity of As. Dietary factors can influence toxicity by producing changes in physiological and

biochemical functions, and in body composition. As well, the bioavailability of As can be greatly affected by food particles in the water, as well as by ingested food.

Adsorption of As to food particles not ingested may reduce the concentration of As to which the fish is exposed, while adhesion of As to ingested food particles may increase exposure and, depending on the composition of the food, may alter absorption in the GIT (Webb 1966). Alternatively, restricting food from post yolk-sac stages may increase the sensitivity of the fish to stress, and can affect absorption, metabolism, and excretion.

The effects of feeding on GI tract absorption of As can also be seen in mammals. In a study by Vahter and Norin (1980), laboratory mice were dosed orally with low and high doses of AsIII and AsV. After assessing the As levels in urine, Vahter and Norin (1980) suggested that at low doses AsIII was more extensively absorbed from the GIT but at higher doses the opposite was true and arsenate was well absorbed from the GIT (89% of 4.0 mg As/kg dose recovered in urine). In a separate study by Odanaka *et al.* (1980) urine recovery of only 48.5% of the original dose (5.0 mg As/kg) suggests that much less AsV is absorbed from the GIT. The differences in these studies may be attributed to differences in feeding regimens given that Vahter and Norin (1980) withheld food from the mice in their study for 2 hr before and 48 hrs after dosing, whereas, in the study by Odanaka *et al.* (1980), food was not restricted.

For the present study, 96 hr exposures to AsIII or AsV were carried out using zebrafish larvae beginning at 72 hpf. The exposures encompassed the whole yolk sac stage (comparable to the alevin stage of other species), therefore, feeding was not a factor. The water source used had a pH of 8.199-8.225 and was moderately hard (139-

143 mg CaCO₃/L), but well within the range of total hardness reported in the literature cited. Stock solutions were made fresh daily so as to ensure a constant concentration of As and to avoid undue speciation. Median lethal concentrations (LC₅₀'s) were determined to be 772 µM and 1347 µM for AsIII and AsV respectively. The LC₅₀'s reported here are within the range of median lethal effects levels reported for the alevin stage of other fresh water fish (Buhl and Hamilton, 1990; 1991). However, simply comparing reported LC₅₀'s for As is not practical given the variability associated with developmental stage and exposure duration, feeding effects, water chemistry, and inter- and intra-species differences. As well as the median lethal effects levels, the present study includes gross effects observed following AsIII and AsV exposure, as well as the relative toxicities of AsIII and AsV. The following section focuses on the gross effects observed in zebrafish following As exposure.

4.3 Gross Toxic Effects of Arsenic to Zebrafish Larvae

Arsenic toxicity has been extensively studied in a variety of vertebrate model systems but there is a paucity of information on the gross abnormalities observed in fish following As exposure. In humans, epidemiological studies describe the systemic effects of As as well as manifestations of gross morphological effects and diseases resulting from this exposure (Engel, *et al.*, 1994; Thomas and Goyer, 1995; Chen, *et al.*, 1995). Many of the effects commonly reported following acute or sub-acute exposure to As, such as edema, paralysis, and death (in severe cases), are the result of damage to vascular and nervous tissue (Webb, 1966). Animal studies describe comparable systemic and morphological effects suggesting that the toxic mode of action of As may be similar in human and non-human vertebrates (Webb, 1966; Nystrom, 1984;

Bekemier and Hirschelmann, 1989; Faires, 2004). Much of the early research on As was carried out in the late 19th and early 20th centuries and this research has since been extensively cited and reviewed in more recent literature. When possible, original studies are cited in the following section, however, more recent reviews of original studies have also been cited when original studies were not accessible.

Unfortunately, published studies on the early life stage toxicity of As in fish focus mainly on mortality, and gross abnormalities were not reported. In the present study, bent body axis in the form of scoliosis and lordosis was observed following AsIII exposure and to a lesser degree in zebrafish larvae exposed to AsV. Blechinger (2002a) also observed trunk abnormalities in zebrafish larvae after exposure to As in the form of sodium arsenite. In mammals, inorganic As has been shown to elicit a number of teratogenic effects including skeletal defects (Willhite, 1981; Who, 2001). Willhite (1981) reported fewer cells in the cephalic mesoderm of hamster embryos isolated from pregnant hamsters exposed to AsV and suggested that damage to the paraxial mesoderm may be associated with the skeletal abnormalities induced by As.

Human and animal studies have reported vascular and nervous tissue effects following As exposure which frequently present in edema, weakness, and partial paralysis. The endothelial lining of blood vessels is particularly sensitive to the action of As and general vascular effects of acute As exposure are increased permeability and dilatation of capillaries resulting in extrusion of plasma into surrounding tissues. Facial, peripheral, and local edema (Webb, 1966), and edema of the gastrointestinal mucosa and various organs (Selby *et al.*, 1977; Faires, 2004) have been reported following acute and chronic exposure to As. Although the chemical form of As is not always known in

accidental exposures, exposure to sodium arsenite and arsenic trioxide (Beckmeir and Hirschelmann, 1989), calcium arsenate (reviewed in Pershagen, 1983), and other As compounds (Kennedy *et al.*, 1986), have been shown to result in vascular damage, and frequently edema, as was observed in this study (Figure 3.1 E,F). Vascular element dilation and edema in the gallbladder and GIT mucosa as well as cardiac and thoracic edema have also been observed in fish following As exposure (Rankin and Dixon, 1994; Pedlar *et al.*, 2002; Blechinger, 2002a).

Nervous system effects are also commonly reported following acute and chronic As exposure. Peripheral neuropathies as well as central nervous system (CNS) effects have been documented in a number of human and animal studies (Danan *et al.*, 1984; Fincher and Koerker, 1987; Morton and Caron, 1989). As-induced peripheral neuropathy is due mainly to axonal degeneration and demyelination of neurons and involves both sensory and motor nerves (Chhuttani *et al.*, 1967). Tingling, numbness, weakness, and paralysis have been reported in humans following acute and chronic exposure to As (Donofrio *et al.*, 1987). Similar effects such as trembling, weakness, staggering gait, and partial paralysis of rear limbs have been reported in domestic animals accidentally exposed to As (Selby *et al.*, 1977). It has also been suggested that As can affect the central nervous system resulting in vertigo, altered mental status, seizures, and toxic encephalopathy (Danan, 1984; Bolla-Wilson and Bleecker, 1987), however, many of these studies involved inhalation of As dust or fumes from industry related pollutants. In fish, nervous system effects may be manifest in erratic swimming and loss of equilibrium (McGeachy and Dixon, 1992). Both erratic swimming and loss

of equilibrium in zebrafish larvae were observed in this study as a result of As exposure (see Section 3.1).

As well as vascular and nervous system effects, high mortality, generally from shock, is often observed as a result of acute exposure to various As compounds (Xie *et al.*, 2004; Nystrom, 1984; Selby *et al.*, 1977) and is a common end point in acute toxicity studies. The lethal effects of As are commonly attributed to destruction of the blood vessels in the lining of the gut, and other organs, which results in decreased blood pressure and subsequent shock (Nystrom, 1984). Multi-organ systems are affected by As and, although liver and kidney failure have been reported in cases of acute exposure, many of the hepatic and renal effects are considered to be secondary to vascular injury (Rosenberg, 1974; Labadie *et al.*, 1990; Fincher and Koerker, 1987). Although vascular and nervous system effects are also observed in fish exposed to As, it has also been suggested that death is due to mucus build up on the gill surface resulting in suffocation as a consequence of inadequate oxygen uptake (Sorensen, 1976). In this study, although underlying causes were not investigated, mortality was observed in zebrafish larvae exposed to AsIII or AsV (Section 3.1).

The gross effects observed and counted in this study were edema, immobility, trunk abnormalities, and mortality. As discussed in the previous paragraphs, edema, trunk abnormalities, immobility, and mortality are consistent with what would be expected given the available information on tissues and organs affected by As; diseases associated with As exposure; and the gross effects observed in humans and animals following As exposure.

Although the effects observed in this study are consistent with what would be expected following acute exposure to As, similar effects have also been observed in zebrafish larvae following exposure to a number of other xenobiotics. In a study by Henry *et al.* (1997), pericardial and yolk sac edema were observed in zebrafish larvae exposed to TCDD. Bent body axis, immobility, and edema were also observed in zebrafish larvae following exposure to Cd (Blechinger *et al.*, 2002a,c), lindane, atrazine, and deltamethrin (Gorge and Nagel, 1990), and triphenyltin acetate (TPTA) (Strmac and Braunbeck, 1999). The similarity of effects observed following exposure to a variety of toxicants suggests that the effects may be an unspecified reaction to xenobiotics rather than specific to As.

4.4 Relative Toxicity of Arsenite and Arsenate

In natural aqueous environments, As exists mainly in the trivalent and pentavalent states, therefore, these species are of most interest when considering toxicity. It is generally accepted that the toxicity of AsIII is greater than that of AsV when exposure conditions are similar. However, at equivalent intracellular levels, AsIII and AsV are considered to be equipotent (ATSDR, 2000). The relative toxicity of an arsenical may depend on a number of factors including inorganic or organic form, solubility, physical state and purity, and rates of absorption and elimination (ATSDR, 2000), but it is largely the valence state that determines the physiological and biological availability and toxicity. The primary mode of AsIII toxicity is through reactions with sulfhydryl groups of proteins and subsequent enzyme inhibition while AsV does not readily react with sulfhydryl groups but exerts its toxicity by competing with phosphate in glycolysis, thereby uncoupling oxidative phosphorylation. However, the toxicity of

AsV is largely a result of its reduction to AsIII, and the greater apparent toxicity of AsIII is likely due to greater cellular uptake and differences in accumulation (Sorensen *et al.*, 1979; Sorensen, 1991). In this study I examined the relative toxicity of AsIII and AsV and found that, as in other fish model systems, AsIII is more toxic to zebrafish than AsV. Also, the present study demonstrated that the EC₅₀ is a somewhat more sensitive indicator of toxicity than the LC₅₀ for both AsIII and AsV.

A wide range of LC₅₀'s have been determined for fish exposed to AsIII or AsV but few studies have examined both valence states concurrently under the same experimental conditions. Reported 96 hr LC₅₀'s in fish range from 3.38 mg As/L (45 µM) to 91 mg As/L (959 µM) for AsIII, and 10.3 mg As/L (137 µM) to >360 mg As/L (>4805 µM) for AsV, depending on the life stage, size, exposure conditions, and species (WHO, 2001). In separate studies, Buhl and Hamilton (1990, 1991) exposed alevin and juvenile stage salmonids to AsIII and AsV as well as a number of other inorganics. When the LC₅₀'s from exposures using fish of similar size, life stage, and species are compared between the two studies, AsIII appears to be 2.4X, 3.4X and 4.2X more toxic than AsV in coho salmon, Arctic grayling, and rainbow trout respectively. In a study that examined AsIII and AsV toxicity concomitantly, Suhendrayatna *et al.* (2002) exposed Japanese medaka (*Oryzias latipes*) to sodium arsenite and sodium arsenate under identical experimental conditions. They reported a 2.07X greater sensitivity to AsIII in these fish than to AsV. This was calculated from 7-day LC₅₀ values of 14.6 mg As/l (194.9 µM) for AsIII and 30.3 mg As/l (404.4 µM) for AsV. In the present study, median lethal concentrations of AsIII and AsV were determined following concomitant exposures of zebrafish larvae (72 hpf) to AsIII and AsV. The calculated 96 hr EC₅₀

(570 μ M) and LC₅₀ (772 μ M) of AsIII were determined to be 2.06X and 1.74X greater respectively, than that of the EC₅₀ (1172 μ M) and LC₅₀ (1347 μ M) of AsV. Thus, the median lethal effects values and differences in sensitivity are consistent with what is reported in the current literature.

Published studies report a wide range of 96 hr LC₅₀'s in fish exposed to AsIII or AsV although few include the median effects concentrations for As. Blechinger (2002a) exposed zebrafish larvae to AsIII employing similar test conditions as the present study, and determined that the EC₅₀ (896 μ M) was 1.73X more sensitive than the LC₅₀ (1552 μ M). My data also indicates that the toxic effects concentration for multiple endpoints (EC₅₀) is a somewhat more sensitive indicator of acute toxicity for AsIII, as well as for AsV, than lethality (LC₅₀) levels alone (Table 4.1) although the difference in sensitivity was relatively low (1.35X and 1.15X more sensitive for AsIII and AsV respectively). Figure 3.2 and 3.3 show a left shift in the regression line for the EC₅₀ when compared to the regression line for mortality alone. Although there is some difference in sensitivity, AsIII and AsV generally accumulate and cause effects in the same target organs/tissues. Therefore they are discussed collectively in the following section.

Table 4.1 Relative toxicity of arsenite and arsenate

	EC ₅₀ (μ M)	LC ₅₀ (μ M)	Relative Sensitivity of EC ₅₀ vs LC ₅₀
AsIII	570	772	1.35
AsV	1172	1347	1.15
Relative toxicity of AsIII vs AsV	2.06	1.74	

4.5 Induction of Heat Shock Protein 70 and Heat Shock Protein 70-Enhanced Green Fluorescent Protein by Arsenic

Previous work in our lab by Blechinger *et al.* (2002c), established background or normal expression patterns of *hsp70* in developing zebrafish larvae. It was determined that over the first 96 hours of development *hsp70* is constitutively expressed in the developing lens from about 28-42 hpf. Expression of *hsp70* was not detected in any other tissues during this period of development. A 1 hr heat shock at 37°C resulted in stress-induced upregulation of *hsp70* throughout the larvae at all stages.

Patterns of *hsp70*-eGFP expression were also determined in transgenic zebrafish over the same developmental stages to determine if the *hsp70*-eGFP reporter gene accurately represents expression of endogenous *hsp70* under non-stressed conditions and following heat shock (Blechinger *et al.*, 2002c). It was determined that tissue-specific expression of *hsp70*-eGFP was identical to that of endogenous *hsp70* during this developmental period. Constitutive lens expression was observed under non-stressed conditions, whereas, expression of GFP in the lens was not evident until about 48 hpf, suggesting that the GFP may need to accumulate before it can be detected. Overall induction of *hsp70*-eGFP throughout the larvae was evident following a one hour heat shock. Expression of *hsp70* and *hsp70*-eGFP were confirmed in wild-type and transgenic larvae at 80 hpf for this study (see section 3.2).

In the present study I demonstrated that *hsp70* and *hsp70*-eGFP induction occurred in the liver, GIT, skin, gills, olfactory rosette, and cardiac region of wild-type and transgenic zebrafish larvae exposed to As. Whole mount photos indicate a dose-dependent increase in the number of cells and tissues expressing *hsp70* (Figure 3.5 and

3.8) and *hsp70*-eGFP (Figure 3.7 and 3.10) in larvae exposed to various concentrations of AsIII and AsV respectively. Particular cells and tissues expressing *hsp70* are more clearly seen in serial sections of larvae (Figure 3.6 and 3.9). Available information on the kinetics and toxicodynamics of As indicates that the organs/tissues shown to express *hsp70*/*hsp70*-eGFP in this study are likely targets for As (Liao *et al.*, 2004; Tsai and Liao, 2006). As well, they are consistent with those shown to accumulate As and those reported to exhibit toxic effects following As exposure in the current literature (Sorensen 1979; Dyer *et al.*, 1993; Rankin and Dixon, 1994; Pedlar and Klaverkamp, 2002). Although diverse exposure regimes, such as dietary vs water borne, may influence the sensitivity of a particular organ/tissue and the degree of accumulation, it is generally agreed that target tissues for accumulation of As are: GIT, liver, skin, gills, kidney, gallbladder, muscle, and spleen.

High levels of sulfhydryl groups (-SH) and direct exposure to water borne toxicants render the skin and gills likely targets for As accumulation and toxicity. In this study, induction of *hsp70*/*hsp70*-eGFP was observed in 100% of larvae exposed to the highest concentrations of AsIII (2000 μ M) and AsV (7500 μ M). Induction also occurred in larvae exposed to lower concentrations of AsIII or AsV but to a lesser degree. The induction of *hsp70* in the gills subsequent to As exposure likely indicates a stress condition for this tissue. Furthermore, protein conformation would be affected as a result of arsenic's affinity for sulfhydryl groups and consequently *hsp70* would be induced.

Gill epithelium is a major route of biouptake for metals such as iron (Bury and Grosell, 2003), Cd and zinc (Zia and McDonald, 1994), and copper (Grosell and Wood,

2002). The accumulation and toxicity of As in fish gills has also been demonstrated (Liao *et al.*, 2004; Tsai and Liao, 2006) and it has been suggested that the cause of death in acute exposures is due to sloughing of the gill epithelium and mucus build up on the gill surface causing suffocation (Sorensen, 1974; 1976). In an effort to estimate the propensity of gill tissue to accumulate As as a result of water borne exposure, Chen and Liao (2004) determined bioconcentration factor (BCF) values in tissues of tilapia exposed to 1 mg As L⁻¹. The BCF relates the concentration of metals in the water to the concentrations in the gills (or other tissues) at equilibrium (Chen and Liao, 2004). Following a 7 day exposure to water borne AsIII they reported a BCF value of 3.21 for tilapia gill tissue. As well, in a similar study, Liao *et al.* (2004) reported a BCF value 2.44 for tilapia gills following a 7 day exposure to AsIII. In both studies the calculated BCF was >1 indicating the potential of the gill to accumulate As following water borne exposure.

In this study, *hsp70* induction was observed in the skin of larvae exposed to even the lowest concentration used (700 µM AsIII and 1500 µM AsV). Induction of *hsp70/hsp70-eGFP* was evident in the skin of 100% of larvae exposed to the highest concentrations of AsIII and AsV (2000 µM and 7500 µM respectively). The high levels of -SH containing proteins in the skin make it a likely target for As accumulation and toxicity. In humans, skin lesions characterized by hyperpigmentation, hyperkeratosis, hypopigmentation, and skin tumors are common symptoms of chronic oral exposure to As (Cebrian *et al.*, 1983; Mazumder *et al.*, 1998). The accumulation of As in fish skin following water borne As has also been demonstrated in a number of studies (Oladimeji *et al.*, 1984; Takatsu *et al.*, 1999). Oladimeji *et al.* (1984), reported accumulation of As

in the skin of rainbow trout chronically exposed to As and suggested the As may be bound to -SH groups in the skin. Takatsu *et al.* (1999), also found relatively high levels of As in the skin of fresh water fish collected from a lake naturally contaminated with As from volcanic activities. However, the concentration of As was higher in epaxial skin (2.5 µg/g wet weight) than in hypaxial skin (0.57 µg/g). They postulated that As may be associated with zinc and, possibly zinc metabolism, since the levels of both were positively correlated in fish organs including skin (correlation coefficient $r = 0.944$).

Dietary exposure to As has also been shown to result in increased levels in fish gills and skin (Pedlar and Klaverkamp, 2002). Lake whitefish (*Coregonus clupeaformis*) fed diets contaminated with various concentrations of AsV for 10, 30, and 64 days showed increased levels of As in a number of tissues including gills and skin. Significant accumulation of As in the skin was reported in fish fed 100 µg As/g food for 30 and 64 days as compared to control groups and, although they did not conduct statistical analyses for the gills, increased concentrations were observed in this tissue as well. The accumulation of As in the skin and gills following dietary exposure may reflect an excretory role for these tissues.

As well as As accumulation, induction of stress proteins in the gills/skin has been demonstrated following exposure of fish to As. Induction of *hsp70* was demonstrated in the gills of whole zebrafish larvae following exposure to water borne AsIII (Blechinger, 2002a). A three hour exposure to 4004 µM sodium arsenite resulted in *hsp70* induction in the skin and gills of 100% of zebrafish larvae exposed. Dyer *et al.*, (1993), reported rapid synthesis of stress proteins in the gills, including those from

the 70 kD family, following acute exposure of fathead minnows to 25 mg As L⁻¹ and determined that even at lower concentrations, more stress proteins (six) were synthesized in the gill than in muscle (five). The differences in stress protein synthesis may have been due to the direct exposure of the gills to the water borne As and/or a higher sensitivity of the gills to environmental changes (Dyer *et al.*, 1993).

Like the skin and gills, the GIT is also directly exposed to toxicants and is, therefore, a target for accumulation and toxicity. Soluble forms of As, such as sodium arsenite and sodium arsenate, are well absorbed via the GI tract (>80%) in both animals and humans (ATSDR, 2000) and fish (Sorensen *et al.*, 1979; Pedlar *et al.*, 2002a; Liao *et al.*, 2004). Pedlar *et al.* (2002a) reported accumulation of As in the GIT of two species of fresh water fish following exposure to dietary As. Significant accumulation of As was observed in the stomach and intestine of both lake whitefish and lake trout exposed to disodium arsenate for 20 days in the diet. Histological alterations, including an increase in goblet cells in the epithelium and increased width of the lamina propria, were also observed in the intestines of lake white fish fed the highest concentration (1000 µg As/g) for 20 days. In an effort to provide further knowledge of organ-specific toxicokinetics and distribution of As in tilapia exposed to water borne As, Liao *et al.*, (2004), conducted a 7-day exposure using sodium arsenite. Fish were sacrificed at various times over 7 days and target organs were dissected out and analyzed for As. They determined toxicokinetic parameters for As in 5 target organs including gill, liver, muscle, intestine, and stomach. All organs tested had BCF values >1 showing the potential to accumulate As when exposed to water borne As. Of the organs tested, however, the stomach and intestine had the highest uptake rate constants (1.53 and 0.67

ml g⁻¹d⁻¹ respectively) and the highest BCF's (9.56 and 4.19 ml g⁻¹ respectively) indicating a greater ability to accumulate As.

Once As is absorbed it enters the circulation and is rapidly distributed to other organs/tissues (Klaassen, 1974; Cockell *et al.*, 1992). Methylation of As occurs mainly in the liver and, until recently, was proposed to be a detoxification process. Although recent studies indicate this may not be the case (Styblo *et al.*, 2000; Petrick *et al.*, 2000; 2001), the liver is still considered a major target organ for As accumulation. In this study, liver expression of *hsp70/hsp70*-eGFP was observed in larvae exposed to low, mid, and high concentrations of AsIII and AsV (Table 3.1 and 3.2). In larvae exposed to the highest concentrations (2000 µM AsIII; 7500 µM AsV) induction occurred in the livers of >50% of exposed larvae. This suggests that the liver is a target organ for As in fish as it is in mammals.

A vast number of effects have been demonstrated in the liver following As exposure and it is considered an important target organ of As toxicity in humans and animals (Lu *et al.*, 2001; Xie *et al.*, 2004; Bashir *et al.*, 2006). Pathophysiologic and molecular effects in hepatic tissue of mice following exposure to various forms of As, including sodium arsenite, were demonstrated by Xie *et al.* (2004). Subchronic exposure to AsIII resulted in dose-dependent accumulation of As in the liver, hepatic global DNA hypomethylation, alterations in gene expression of a number of genes (including stress-related genes), and variable other toxic effects. A chronic six month study by Bashir *et al.* (2006) revealed a variety of adverse effects in the rat liver following exposure to As. In addition to significant increases in cytochrome-P450 and lipid peroxidation, reductions in glutathione levels, and post-mitotic apoptosis, they

reported hepatocyte degeneration, focal necrosis, and chronic inflammatory infiltrate, all suggesting hepatocellular damage. Similarly, As related toxic effects such as fatty infiltration, cirrhosis, central and focal necrosis in the livers of fish have also been described (Sorensen, *et al.*, 1980), as well as As accumulation in the liver of fish (Liao *et al.*, 2004; Tsai and Liao, 2006) and stress protein induction in fish hepatocytes following As exposure (Boone and Vijayan, 2002).

Although biomagnification of As does not appear to occur in the aquatic food chain, a number of studies indicate that bioaccumulation of As does occur in fish organs/tissues and, like mammals, the liver is a target organ for As accumulation and toxicity (Sorensen, 1980; Liao *et al.*, 2004; Tsai and Liao, 2006). Tsai and Liao (2006) examined the accumulation ability of As in target organs/tissues of tilapia including gills, carcass, alimentary canal, and liver following water borne exposure to AsIII. The organ-specific uptake rate constant for the liver was determined to be $0.61 \text{ mL g}^{-1} \text{ d}^{-1}$. Although this was slightly lower than that of the alimentary canal ($0.84 \text{ mL g}^{-1} \text{ d}^{-1}$), it was 2X higher than that of the gill and 5X that of the carcass (0.31 and $0.12 \text{ mL g}^{-1} \text{ d}^{-1}$ respectively). In an earlier study in the same lab, Liao *et al.*, (2004), also examined the uptake and accumulation of As by various target organs and reported the uptake ability of the liver was higher than that of the gills and muscle but somewhat lower than the GIT. The accumulation of As in the liver is mirrored in field studies where As concentration in hepatic tissue has been reported to be significantly higher than that of other tissues in fish exposed to As in the environment (Mahar *et al.*, 1999; Burger *et al.*, 2004). These studies demonstrate the high uptake and high accumulating potential of liver for As III. The accumulation of As in hepatic tissue of fish is likely a result of the

vital role the liver plays in detoxification, however, the elevated levels in the liver may be partially attributed to enterohepatic redistribution (Sorensen, 1991).

The induction of stress proteins, including *hsp70*, has been demonstrated in fish hepatocyte cell lines following AsIII exposure (Boone and Vijayan, 2002), and in the livers of whole fish exposed to AsIII (Blechinger, 2002a). Using primary cultures of rainbow trout hepatocytes Boone and Vijayan (2002) analyzed changes in stress protein expression following a 4 hr exposure to 50 μ M AsIII. Although there were no changes observed in the expression of *hsc70* (a constitutively expressed stress protein) as a result of the exposure, *hsp70* accumulation was found to be significantly higher following exposure to AsIII. AsIII exposure has also been linked to increased accumulation of *hsp70* in hepatic tissue of whole fish following water borne exposure. Following exposure of whole zebrafish larvae to 4004 μ M AsIII, Blechinger (2002a) found significant increases in *hsp70* mRNA in a number of tissues/organs, including the liver. In the present study, induction of *hsp70* was evident in 27/44 larvae exposed to the highest concentration of AsIII (2000 μ M). The information in the literature cited above indicates that this induction is likely due to the cytotoxicity of As. However, the proportion of larvae expressing *hsp70* in the liver was relatively low compared to other tissues. This may be due in part to the three hour exposure duration and the kinetics of As. The short duration may result in lesser amounts of As reaching the liver and, being the site of methylation, enzymes in the liver may provide some protection from AsIII resulting in a lower sensitivity.

I also observed *hsp70-eGFP* expression in the cardiac region of transgenic larvae and, more specifically, *hsp70* induction occurred in the pericardial muscle of

wild-type zebrafish larvae following exposure to AsIII or AsV. There is little information in current literature on the cardiac effects of As in fish. However, human and animal model studies indicate cardiac tissue accumulation and adverse effects following As exposure. In a study by Wu *et al.*, (2003) significant accumulation of As in the cardiac muscle of rabbits was demonstrated following parenteral administration of arsenic trioxide (As₂O₃). Although no significant changes were observed in the heart tissue of rabbits acutely treated with low doses (1 to 30 µM) of As, electrophysiological disturbances such as ventricular arrhythmia and prolonged QT intervals occurred in rabbits treated with 300 µM and in those chronically exposed to 30 µM As₂O₃. Histopathology revealed no pathological changes in the cardiac tissue of any of the exposed rabbits. Accumulation of As in cardiac muscle has also been demonstrated in chickens exposed orally in drinking water (Pizarro *et al.*, 2004) and cardiac disturbances have been observed in mice dosed intraperitoneally with As₂O₃ (Li *et al.*, 2002). However, along with functional alterations, cardiomyopathy and myocardial apoptosis were also reported. Electrophysiological changes and cardiomyopathies have also been observed in humans during following accidental or therapeutic exposure to As. Clinical studies of promyelocytic leukemia patients treated intravenously with As₂O₃ reported a number of cardiac disturbances including prolonged QT intervals, ventricular tachycardia, and in one patient who subsequently died, complete atrial-ventricular block (Huang *et al.*, 1998). A postmortem examination of this patient revealed abnormally high levels of As in the cardiac tissue. As well, in a postmortem examination of a patient who had been chronically exposed to As and who subsequently died from a fatal cardiac event, Hall and Harruff (1989) reported evidence

of interstitial myocarditis. These studies suggest that cardiac tissue is a target for As toxicity and may explain why I detected *hsp70/hsp70*-eGFP in the cardiac region of the zebrafish larvae exposed to As in this study.

Morphological integrity did not allow for definitive determination of expression of *hsp70* in olfactory tissues of wild-type larvae. Therefore, olfactory region expression was not included in counts. However, induction of *hsp70*-eGFP was evident in the olfactory region of transgenic larvae exposed to the highest (2000 μ M) concentration of AsIII (Fig. 3.7). Arsenic uptake has not been specifically demonstrated in the olfactory tissue of fish, however, in a similar study to the present one, *hsp70* induction in the olfactory region was observed in wild-type zebrafish larvae following exposure to 4004 μ M AsIII (Blechinger 2002a). Also, Rankin and Dixon (1994) observed olfactory and mandibular necrosis in tilapia following acute and chronic water borne exposure to AsIII. They reported that the feeding response of fish exposed to the highest concentration (9.64 mg AsL⁻¹), was noticeably affected and may have been associated with the rostral lesions and a subsequent loss of chemoreception. They also reported a positive correlation between mortality and the occurrence of the rostral lesions over time. Also, a number of studies have demonstrated the accumulation and/or toxic effects of other metals such as Cd (Scott *et al.*, 2003) and copper (Julliard *et al.*, 1996) in the olfactory region of fish.

The uptake and/or accumulation of various metals as well as observed *hsp70* induction and toxic effects following exposure to AsIII and AsV in the above mentioned studies (Section 4.4), suggest that the *hsp70*-eGFP expression observed in the present study was likely due to As toxicity.

5.0 Future Direction

In this study a clear dose-response relationship was demonstrated for zebrafish larvae exposed to As, however, adverse effects and *hsp70* induction were evident only at high levels. The concentrations required for *hsp70* induction suggests that the fish are not accumulating As. The resistance of some species of fish to As may be due to a combination of uptake, elimination and storage processes.

Phosphate transporters have been demonstrated to participate in AsV uptake in prokaryotes (*Escherichia coli*) as well as eukaryotes (*Saccharomyces cerevisiae*) (Rosen, 2002). Although it has not been demonstrated, it is likely that AsV is taken up similarly in mammals. Aquaglyceroporin, a member of the aquaporin superfamily, have been demonstrated to be the route of uptake of AsIII in *Saccharomyces cerevisiae* (Wysocki *et al.*, 2001) and, more recently, in mammals (Liu *et al.*, 2002). As well as uptake pathways, extrusion systems such as MRP's (multidrug resistance-associated proteins) have been investigated and have been demonstrated to confer As resistance in eukaryotic organisms (Rosen, 2002). Further investigation into these uptake and extrusion pathways of As in fish may help to elucidate the apparent resistance of some species.

6.0 References

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Appendix 1 Endpoint Counts For the 96hr Acute Toxicity Test For Arsenite on Zebrafish Larvae

			Mortality			Trunk Abnormality			Edema			Immobility			Multiple Endpoints		
dose uM	rep	count	rep%	avg %	SEM	count	rep%	avg %	SEM	count	rep%	avg %	SEM	count	rep%	avg %	SEM
0	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
	3	0	0.0			0	0.0			0	0.0			0	0.0		
10	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
	3	0	0.0			0	0.0			0	0.0			0	0.0		
100	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
	3	0	0.0			0	0.0			0	0.0			0	0.0		
300	1	0	0.0			0	0.0			1	5.0			0	0.0		
	2	1	5.0	1.7	1.7	0	0.0	2.5	1.7	0	0.0	0.0	0.0	0	0.0	5.0	0.0
	3	0	0.0			1	5.0			0	0.0			0	0.0	5.0	
400	1	2	10.0			0	0.0			0	0.0			0	0.0		
	2	2	10.0	11.7	1.7	0	0.0	2.5	1.7	0	0.0	0.0	0.0	1	5.0	1.7	1.7
	3	3	15.0			1	5.0			0	0.0			0	0.0	20.0	
500	1	4	20.0			1	5.0			0	0.0			1	5.0		
	2	3	15.0	16.7	1.7	0	0.0	7.5	2.9	0	0.0	2.5	1.7	2	10.0	6.7	1.7
	3	3	15.0			2	10.0			1	5.0			1	5.0	7	35.0
1000	1	15	75.0			1	5.0			1	5.0			1	5.0	18	95.0
	2	15	75.0	76.7	1.7	0	0.0	5.0	1.7	2	10.0	10.0	2.9	1	5.0	18	100.0
	3	16	80.0			1	5.0			3	15.0			0	0.0	20	100.0
2000	1	20	100.0			0	0.0			0	0.0			0	0.0		
	2	19	95.0	96.7	1.7	0	0.0	0.0	0.0	0	0.0	0.0	0.0	1	5.0	3.3	1.7
	3	19	95.0			0	0.0			0	0.0			1	5.0	20	100.0
3000	1	20	100.0			0	0.0			0	0.0			0	0.0		
	2	20	100.0	100.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	20	100.0
	3	20	100.0			0	0.0			0	0.0			0	0.0	20	100.0

Each replicate consisted of 20 larvae for a total of 60 per treatment. The number of larvae positive for a given endpoint is listed in the # **response** column. The % **response** is the number of positives expressed as a percentage. The average of the three replicates and the standard error of the mean are listed under the **avg %** and **SEM** columns respectively. Multiple endpoints include mortality, trunk abnormalities, edema, and immobility.

Appendix 2 Endpoint Counts For the 96hr Acute Toxicity Test For Arsenate on Zebrafish Larvae

			Mortality			Trunk Abnormality			Edema			Immobility			Multiple Endpoints		
dose uM	rep	count	rep%	avg %	SEM	count	rep%	avg %	SEM	count	rep%	avg %	SEM	count	rep%	avg %	SEM
0	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
	3	0	0.0			0	0.0			0	0.0			0	0.0		
100	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0
	3	0	0.0			0	0.0			0	0.0			0	0.0		
500	1	0	0.0			0	0.0			0	0.0			0	0.0		
	2	1	5.0	3.3	1.7	0	0.0	0.0	0.0	0	0.0	0.0	0.0	0	5.0	3.3	1.7
	3	1	5.0			0	0.0			0	0.0			1	5.0		
750	1	2	10.0			0	0.0			0	0.0			3	15.0		
	2	2	10.0	10.0	0.0	0	0.0	0.0	0.0	1	5.0	3.3	1.7	3	15.0	13.3	1.7
	3	2	10.0			0	0.0			0	0.0			2	10.0		
1000	1	6	30.0			1	5.0			0	0.0			8	40.0		
	2	6	30.0	31.7	1.7	0	0.0	2.5	1.7	0	0.0	0.0	3.3	8	40.0	38.3	1.7
	3	7	35.0			0	0.0			0	0.0			7	35.0		
1500	1	16	80.0			1	5.0			1	5.0			19	95.0		
	2	13	65.0	65.0	8.7	2	10.0	2.5	2.9	1	5.0	5.0	0.0	18	90.0	81.7	10.9
	3	10	50.0			0	0.0			1	5.0			12	60.0		
2500	1	18	90.0			0	0.0			0	0.0			19	95.0		
	2	20	100.0	95.0	2.9	0	0.0	0.0	0.0	0	0.0	0.0	0.0	20	100.0	98.3	1.7
	3	19	95.0			0	0.0			1	5.0			20	100.0		
5000	1	20	100.0			0	0.0			0	0.0			20	100.0		
	2	18	90.0	96.7	3.3	0	0.0	0.0	0.0	1	5.0	1.7	1.7	19	95.0	98.3	1.7
	3	20	100.0			0	0.0			0	0.0			20	100.0		
7500	1	20	100.0			0	0.0			0	0.0			20	100.0		
	2	20	100.0	100.0	0.0	0	0.0	0.0	0.0	0	0.0	0.0	0.0	20	100.0	100.0	0.0
	3	20	100.0			0	0.0			0	0.0			20	100.0		

Each replicate consisted of 20 larvae for a total of 60 per treatment. The number of larvae positive for a given endpoint is listed in the # **response** column. The % **response** is the number of positives expressed as a percentage. The average of the three replicates and the standard error of the mean are listed under the **avg %** and **SEM** columns respectively. Multiple endpoints include mortality, trunk abnormalities, edema, and immobility.

Appendix 3 Tissue Counts For Larvae Expressing *hsp70* and *hsp70*-eGFP Following Arsenite Exposure and Fisher's Exact Test Probabilities

Larvae Type	Concentration	Tissue Expressing	Treated		Control		Probability Value to Accept Null Hypothesis
			+	-	+	-	
Transgenic	2000uM	liver	12	2	0	10	3.97704E-05
		GIT	13	1	0	10	6011853E-06
		cardiac	11	3	0	10	0.000185595
		gills/skin	14	0	0	10	5.09877E-07
	1000uM	liver	11	3	0	10	0.00018559
		GIT	12	2	0	10	2.3623E-05
		cardiac	8	6	0	10	0.006310242
		gills/skin	10	4	0	10	0.000695983
	700uM	liver	7	8	0	10	0.02312253
		GIT	7	8	0	10	0.02312253
		gills/skin	5	10	0	10	p > 0.05
		liver	27	17	0	45	1.95127E-11
Wild-type	2000uM	GIT	44	0	0	45	1.92627E-26
		pc muscle	42	2	0	45	2.17284E-23
		gills/skin	44	0	0	45	1.92627E-26
		liver	21	24	0	45	3.37923E-08
	1000uM	GIT	27	18	0	45	3.46892E-08
		pc muscle	18	27	0	45	7.18545E-07
		gills/skin	30	15	0	45	6.79397E-13
		liver	7	38	0	45	0.011091281
	700uM	GIT	9	36	0	45	0.00223671
		gills/skin	4	41	0	45	p>0.05

Larvae per treatment group that were positive for expression of *hsp70* or *hsp70*-eGFP in a given tissue were counted and compared to control groups. The Fisher's Exact probability was calculated for each. Tissue expression in wild-type larvae was determined from serial section and tissues expressing the reporter gene construct in transgenic larvae were assessed in live larvae observed under a fluorescence microscope. pc muscle, pericardial muscle; GIT, gastrointestinal tract epithelium.

Appendix 4 Tissue Counts For Larvae Expressing *hsp70* and *hsp70*-eGFP Following Exposure To Arsenate and Fisher's Exact Test Probabilities

Larvae Type	Concentration	Tissue Expressing	Treated		Control		Probability Value to Accept Null Hypothesis
			-	+	+	-	
Transgenic	7500uM	liver	13	1	0	10	6.11853E-06
		GIT	14	0	0	10	5.09877E-07
		cardiac	11	3	0	10	0.000185595
		gills/skin	14	0	0	10	5.09877E-07
	2500uM	liver	9	6	0	10	0.003786145
		GIT	9	6	0	10	0.003786145
		cardiac	7	8	0	10	0.02312253
		gills/skin	10	5	0	10	0.001336287
	1500uM	liver	7	8	0	10	0.02312253
		GIT	5	10	0	10	p > 0.05
		gills/skin	3	12	0	10	p > 0.05
		liver	25	20	0	45	3.91623E-10
Wild-type	7500uM	GIT	30	15	0	45	6.79397E-13
		pc muscle	37	8	0	45	1.00211E-17
		gills/skin	45	0	0	45	9.63137E-27
		liver	19	26	0	44	3.39587E-07
	2500uM	GIT	22	23	0	44	1.54479E-08
		pc muscle	23	22	0	44	5.22503E-09
		gills/skin	27	18	0	44	4.98657E-11
		liver	13	30	0	45	5.03328E05
	1500uM	GIT	13	30	0	45	5.03328E-05
		gills/skin	9	34	0	45	0.001816519

Larvae per treatment group that were positive for expression of *hsp70* or *hsp70*-eGFP in a given tissue were counted and compared to control groups. The Fisher's Exact probability was calculated for each. Tissue expression in wild-type larvae was determined from serial section and tissues expressing the reporter gene construct in transgenic larvae were assessed in live larvae observed under a fluorescence microscope. pc muscle, pericardial muscle; GIT, gastrointestinal tract epithelium.